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Award Number: DAMD17-97-1-7035

TITLE: Molecular Mechanism of Action of Genistein and Related Phytoestrogens in Estrogen Receptor Dependent and Independent Growth of Breast Cancer Cells

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REPORT DATE: July 2000

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	July 2000	Final (1 Jul 97 - 30 Jun 00)

4. TITLE AND SUBTITLE
Molecular Mechanism of Action of Genistein and Related Phytoestrogens in Estrogen Receptor Dependent and Independent Growth of Breast Cancer Cells

5. FUNDING NUMBERS
DAMD17-97-1-7035

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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES
Report contains color photos

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT ***(Maximum 200 Words)***

During the 3 years of my pre-doctoral research supported by the U.S. Army's Breast Cancer Research Program, I studied estrogen receptor (ER)-dependent and -independent effects of five structurally related phytoestrogens on breast cancer cell growth and apoptosis. Our studies showed that genistein binds to the ER with lower affinity compared to estradiol. However, genistein-bound ER is a 4S monomeric protein, while estradiol-bound ER is multimeric in our sucrose density gradient experiments. We also showed that genistein-ERβ complex bound to the consensus estrogen responsive element (ERE) with high affinity. In contrast, genistein bound to ERα did not significantly bind to the ERE. These results suggest that genistein and estradiol exert different effects on ER-mediated functions.

In ER-negative cells, genistein and quercetin inhibited cell growth and induced apoptosis in a dose-dependent manner. Daidzein, kaempferol and biochanin A were less effective. The mechanism of growth inhibition in ER-positive and ER-negative breast cancer cells by genistein and quercetin included G2/M cell cycle arrest and alterations in cyclin B1 protein. Our results suggest that genistein and quercetin may be developed as chemotherapeutic agents against treatment of ER-negative tumors.

14. SUBJECT TERMS
Breast Cancer

15. NUMBER OF PAGES
62

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

NSN 7540-01-280-5500

Standard Form 298 (Rev. 2-89)
Prescribed by ANSI Std. Z39-18
298-102

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Sridevi B 7-28-2000

PI - Signature Date

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(5) INTRODUCTION

Phytoestrogens are plant compounds that have structural similarity to the endogenous female sex hormone, estradiol (E_2) and are thought to confer protective effects against breast cancer in vegetarians (1, 2, 3). A major phytoestrogen in soy, genistein has also been reported to have weak estrogenic effects in the reproductive systems of animals and MCF-7 breast cancer cells (4, 5). E_2 is considered a major risk factor in breast tumor development and progression. E_2 binds to the estrogen receptors, ERα and ERβ and the receptor-ligand dimers bind to the consensus estrogen responsive elements (ERE) in the promoter region of genes involved in cell growth and enhance their transcription (6). Therefore, a better understanding of the mechanism of action of phytoestrogens in breast cancer cells will help determine their potential harmful or beneficial effects in chemoprevention as well as their usefulness in breast cancer therapy. My proposed hypotheses were briefly:

"Genistein and related phytoestrogens can exert estrogenic effects utilizing the ER pathway or type-II estrogen binding sites (EBS). Interaction of genistein with ER can induce a conformational change in ER that is different from that of E_2-bound ER. Growth stimulatory and inhibitory effects of phytoestrogens are intimately linked to their effects on cell cycle distribution and cell cycle regulatory proteins."

During the past 3 years of my pre-doctoral research supported by the U.S. Army's Breast Cancer Research Program, we studied the ER-based actions of genistein by determining the binding affinities of genistein to ER using cellular and recombinant ERα. To understand how the molecular structure of genistein is associated with estrogenic properties, we compared it with other structurally related compounds such as daidzein, biochanin A, quercetin and kaempferol in ER binding. Furthermore, to differentiate the mechanism of action of genistein from that of estradiol, we used [^{14}C]-labeled genistein and measured its interaction with ER from MCF-7 cells and human recombinant ERα and ERβ, using sucrose density gradient analysis. We also examined ER-ERE interactions in the presence of genistein and estradiol by electrophoretic mobility shift assays (EMSA). The results from these studies have been submitted as a manuscript for publication (COPY ATTACHED).

To understand the mechanism of growth inhibition by phytoestrogens, we studied their effects on cell growth, cell cycle kinetics, cell cycle regulatory proteins and apoptosis of ER-negative MDA-MB-468 breast cancer cells. The results from these studies have been published (REPRINT ATTACHED).

(7) KEY RESEARCH ACCOMPLISHMENTS

A summary of my accomplishments during the 3 years of pre-doctoral support from the US Army is given below.

Tasks for the final report	Status
• Months 1-12	Binding studies to determine affinities of phytoestrogens with ER and type-II EBS and circular dichroism studies to examine conformational changes in phytoestrogen-bound ER- completed and the results from extension of the conformation studies are submitted as a manuscript (Appendix B).
• Months 13-24	Cell proliferation studies - completed and the results have been published (Appendix A). ER-ERE interactions in the presence of phytoestrogens- completed and the data were presented as an abstract at the Annual Meeting of the American Association of Cancer Research, 1999 Philadelphia. Expression of c-*myc*, ODC and pS2 genes by phytoestrogens- partially completed. Additional experiments in progress.
• Months 25-36	Flow cytometric analysis to determine cell cycle distribution of phytoestrogen-treated breast cancer cells and western blot analysis for cell cycle regulatory proteins- completed (Appendix A).

(8) REPORTABLE OUTCOMES

Manuscripts based on work supported by this award

1. Balabhadrapathruni S, Thomas TJ, Yurkow EJ, Amenta PS, and Thomas T. Effects of genistein and structurally related phytoestrogens on cell cycle kinetics and apoptosis in MDA-MB-468 human breast cancer cells. Oncology Reports 7: 3-12, 2000.

2. Balabhadrapathruni S, Thomas T, Ghosh S, Thomas TJ. Stabilization of cellular and recombinant estrogen receptors as 4S monomeric form in the presence of genistein (Manuscript in revision).

Manuscripts from tasks not proposed

3. Thomas T, Balabhadrapathruni S, Gardner C, Hong J, Faaland C, Thomas TJ. Effects of epidermal growth factor on MDA-MB-468 breast cancer cells: alterations in polyamine biosynthesis and the expression of p21/CIP1/WAF1. J of Cellular Physiol 179: 257-266, 1999.

4. Faaland CA, Thomas TJ, Balabhadrapathruni S, Langer T, Mian S, Shirahata A, Gallo MA, and Thomas T. Molecular correlates of the action of bis(ethyl)polyamines in breast cancer cell growth inhibition and apoptosis. Biochemistry and Cell Biol. (In Press).

5. Thomas TJ, Balabhadrapathruni S, Thomas A, Thomas RM, Shirahata A and Thomas T. Facilitation of triplex DNA formation and breast cancer cell growth inhibition by bis(ethyl)polyamine analogs and a triplex forming oligonucleotide targeted to the P1 promoter of c-myc oncogene. (Manuscript submitted).

Abstracts

1. Balabhadrapathruni S, Thomas TJ, Yurkow EJ, Amenta PS, and Thomas T. Effects of genistein and structurally related phytoestrogens on cell cycle kinetics and apoptosis in MDA-MB-468 human breast cancer cells. Proceedings of the Department of Defense Breast Cancer Research Program Meeting, ERA OF HOPE, pp. 353-354, 2000. (COPY ATTACHED)

2. Farooque P, Balabhadrapathruni S, Thomas TJ, Gallo MA, and Thomas T. Oxidative/reducing agents alter the ligand binding and dimerization of estrogen receptor (ER) alpha and beta. Proceedings of the American Association for Cancer Research, pp. 138, 2000. (COPY ATTACHED).

3. Balabhadrapathruni S, Thomas T, Yurkow E, and Thomas TJ. Genistein induces apoptosis and biphasic changes in cyclin B1 in MDA-MB-468 breast cancer cells. Proceedings of the American Association for Cancer Research, pp. 138, 1999. (COPY ATTACHED).

4. Balabhadrapathruni S, Ghosh S, Thomas TJ, and Thomas T. Interaction of genistein and other phytoestrogens with estrogen receptor. Proceedings of the American Association for Cancer Research, pp. 652, 1999. (COPY ATTACHED).

5. Thomas TJ, Thomas RM, Antony T, Balabhadrapathruni S, Saminathan M, Shirahata A, and Thomas T. Facilitation of triplex DNA formation by bis (ethyl)(BE) polyamine analogs and breast cancer cell growth inhibition by polyamine analogs-complexed triplex forming oligonucleotide (TFO). Proceedings of the American Association for Cancer Research, pp. 19, 1999. (COPY ATTACHED).

6. Balabhadrapathruni S, Thomas T, Yurkow E, and Thomas TJ. Genistein treatment causes G2/M cell cycle arrest and cyclin B1 accumulation in MCF-7 breast cancer cells: A possible mechanism for the preventive action of genistein. Proceedings of the American Association for Cancer Research, pp. 389, 1998. (COPY ATTACHED).

7. Balabhadrapathruni S, Thomas T, Shirahata A, and Thomas TJ. A synergistic antiproliferative effect of a polyamine analogue and a triplex forming oligonucleotide (TFO) on MCF-7 breast cancer cells. Proceedings of the American Association for Cancer Research, pp. 94, 1997. (COPY ATTACHED).

8. Balabhadrapathruni S, Thomas T, and Thomas TJ. Estrogenic and anti-estrogenic actions of genistein in human breast cancer cell growth mediated through the polyamine pathway. Proceedings of the American Association for Cancer Research, pp. 207, 1997. (COPY ATTACHED).
9. Balabhadrapathruni S, Thomas TJ, Gallo M, and Thomas T. Growth inhibitory effects of a telomeric oligonucleotide on MCF-7 breast cancer cells. Proceedings of the American Association for Cancer Research, pp. 505, 1997. (COPY ATTACHED).

Award of the Ph. D degree
My Ph.D thesis work is in the final phase and I expect to submit my thesis in December, 2000.

Awards
Received the "Gallo Award for Outstanding Cancer Research" for my presentation "Genistein treatment causes G2/M cell cycle arrest and cyclin B1 accumulation in breast cancer cells: A possible mechanism for the preventive action of genistein", presented by the Cancer Institute of New Jersey at the 1998 New Jersey Cancer Retreat on May 29, 1998 (COPY ATTACHED).

(6) BODY OF REPORT

A. METHODS

1. Cell Culture and Chemicals. MCF-7 cells are maintained in Dulbecco's Modified Eagles Medium (DMEM) with 100 μg/ml penicillin, 100 μg/ml streptomycin, 2 μg/ml insulin, and 10% fetal bovine serum (FBS). For two weeks before each experiment, cells were grown in phenol-red free DMEM as phenol red has estrogenic effects (7). FBS was treated with dextran coated charcoal (DCC) to remove endogenous estrogens and added to the medium as described previously (8). MDA-MB-468 cell line was obtained from the American Type Culture Collection (ATCC, Manassas, Virginia). The cells were maintained in Improved minimum essential medium (IMEM) with 10% fetal bovine serum (FBS), 4 mM glutamine, 0.4 mM sodium pyruvate, 40 μg/ml gentamycin, and 100 μg/ml each of penicillin and streptomycin.

Genistein, quercetin, daidzein, kaempferol and biochanin A were purchased from Sigma Chemical Co. (St. Louis, MO). Stock solutions were made in dimethylsulfoxide (DMSO), aliquoted, and frozen until use. The APO-BRDU^{TM} assay kit was purchased from Pharmingen (San Diego, CA). The antibodies for cyclin B1, C-terminal domain of cdc2 and β-actin were obtained from Neomarkers (Union City, CA).

2. Competitive binding assay of phytoestrogens with labeled estradiol. To prepare cellular ER, MCF-7 cells from 10 T0150 flasks were harvested into PBS and sonicated in TEDG buffer (10 mM Tris, 1 mM EDTA, (pH 7.5), I mM dithiothreitol (DTT) and 10% glycerol). The cellular homogenate was centrifuged at 100,000 x g to obtain the cytosol. The cytosol (250 μl) was incubated for 3 h at 4 °C with 5 nM [^{3}H]-E_2 and unlabeled phytoestrogen (10^{-5}- 10^{-8} M). After incubation, DCC suspension was added to the

samples to remove free E_2. The samples were then centrifuged at 750 x g for 5 min at 4 ° C . Supernatant was sedimented through a 10-30% sucrose-TEDG buffer. [^{14}C]-labeled bovine serum albumin and γ-globulin were used as sedimentation markers. Gradients were centrifuged in a Beckman SW60 rotor at 53,000 rpm for 16 h. Fractions were collected into 5 ml scintillation fluid and the bound estradiol was counted using a scintillation counter. Nonspecific binding was determined in parallel with the receptor incubated with [^{3}H]-E_2 and 200-fold excess cold E_2. Concentration dependent, competition binding curves were determined for each compound. Results are expressed as percent [^{3}H]-E_2 bound in the absence (100%) and presence of the competitor.

Human recombinant ER, 500 fmol/ml was incubated with 5 nM [^{3}H]-E_2 and different concentrations of the phytoestrogens, and treated as described above for cellular ER. In parallel experiments, receptor was incubated with 5 nM [^{3}H]-E_2 and increasing concentrations of unlabeled E_2 .

3. Type-II EBS analysis in human breast cancer cells. Type-II EBS was measured by a whole cell assay as described by Ranelletti et al (9). MCF-7 and MDA-MB-468 cells (5 x 10^4)/well) were plated into Multiwell plates with the appropriate medium. After 24 h, the cells were refed with fresh medium without serum. The cells were then dosed with increasing concentrations (5-50 nM) of [^{3}H]-E_2 alone or with 200-fold molar excess of diethyl stilbestrol (DES) and incubated for 3 h at 37 °C. After 3 h, the cells were kept on ice for 30 min and washed 4 times in 1 ml volumes of ice cold HBSS. To each well, 1 ml of 100% ethanol was added and incubated at 37 °C. Bound E_2 was measured by a liquid scintillation counter. Competition for type-II EBS was determined by incubating cells with 40 nM [^{3}H]-E_2 and increasing concentrations of one of the phytoestrogens. Radioactivity bound to the cells was determined and IC_{50} values were calculated for each of the compounds, from the concentration dependence of the displacement of [^{3}H]-E_2 from type-II EBS.

4. Circular dichroism measurements. The secondary structural changes in the human recombinant ER (1.5 μM protein) was studied in the presence of genistein, using CD spectroscopy. Genistein (0.5-10 μM) or same concentrations of estradiol were incubated with the ER at room temperature overnight in storage buffer (50 mM Tris, 1 mM sodium vanadate, 1 mM EDTA, 500 mM KCl, 10% glycerol and 2 mM DTT). All CD spectra were recorded using an AVIV 62D spectropolarimeter with a water-jacketed cell of 1mm path length. The spectral wavelength range measured was between 200-260 nm. The cell temperature was kept constant with a circulating water bath at 25 °C. The a-helecity was calculated from the reported value of molar elipticity of a 100% helecal peptide, (θ)(222 nm) = 33,500 deg cm^2 dmol^{-1}.

5. Electrophoretic Mobility Shift Assay to Determine Binding of Genistein-ER Complex with ERE. The consensus sequence of ERE (5' CCAGGTCAGAGTGACCTGAGCTAAAATAACACATTCAG) from vitellogenin gene was purchased from Oligos, Etc. (Wilsonville, OR). The oligonucleotide and its complementary strand were dissolved in 10 mM Tris-HCl (pH 8.0), 200 mM NaCl and equimolar solutions were boiled for 10 min and allowed to anneal for 2 h at room

temperature. Then the oligonucleotide solution was dialyzed thrice against the same buffer and end-labeled with ^{32}P-γ-ATP using an end labeling kit from Promega (Madison, WI). Human recombinant ERα or ERβ, 500 ng each was incubated with different concentrations of genistein for 2 h. About 20,000 cpm of labeled probe and 4x binding buffer were added to the ER-genistein mixture to reach a final concentration of 10 mM Tris-HCl, 10% glycerol, 1 mM dithiothreitol and 10 µg/ml poly(dI-dC).poly(dI-dC), (Pharmacia, Piscataway, NJ). The binding reaction was allowed to proceed for 1 h at 4°C and 30 min at room temperature. Then the samples were loaded in a 6% polyacrylamide gel and electrophoresed for 3 h at 100 V. The gel was dried and exposed to Kodak Biomax MR-1 film for autoradiography for 3 to 6 h. Intensity of the DNA-protein complex was quantified using a scanning densitometer.

6. [^{3}H]-Thymidine Incorporation Assay. Cells (0.5 x 10^6/dish) were plated in 60 mm culture dishes and allowed to adhere for 24 h. The cells were dosed with different concentrations of phytoestrogens for 24, 48 and 72 h. One hour before the treatment time ended, one µCi/ml of [^{3}H]-thymidine was added to the cells. The radioactivity incorporated into the cellular DNA was measured by liquid scintillation counting.

7. Northern Blot Analysis of ODC and c-*myc* mRNA. Total RNA was extracted using the TRI reagent (Molecular Research Inc., Cincinnati, OH). RNA concentration was determined by measuring absorbance at 260 nm and converted to µg/ml, using the equation, one Absorbance unit (A260) = 40 µg/ml. Twenty µg of RNA was loaded into each well and RNA molecules were separated under denaturing conditions on 1% agarose gel, electrophoresed for 24 h. RNA from the gel was transferred onto a nylon membrane (Oncor, Gaithersburg, MD). Prior to hybridization, the membrane was preconditioned with hybrisol for 1 h at 42°C. Overnight hybridization was carried out with cDNA probe for c-*myc* or ODC labeled with ^{32}P-α-dCTP (~10^6 cpm/ml of hybridization solution). The membranes had 3 x 15 min washes in 0.1% SSC/0.1%SDS at room temperature and 1 h in 0.1 X SSC/0.1% SDS at 52°C. Membranes were then exposed to Kodak X-OMAT AR film at -70°C for 48-72 h. Relative intensities of bands were quantified using a scanning densitometer.

8. Flow Cytometric Determination of Cell Cycle Analysis. MCF-7 and MDA-MB-468 cells (2 x 10^6 cells/dish) were allowed to adhere to plate for 24 h and then dosed with genistein, or other phytoestrogens. After 24, 48 or 72 h of treatment, media was removed, 2 ml citrate buffer (40 mM Citrate-Trisodium, 250 mM sucrose and 5% DMSO) added and cells frozen at -70°C until further analysis. For cell cycle analysis, cells were thawed and harvested to collect the pellet. The cell pellet was treated with trypsin for 10 min and trypsin inhibitor and RNase (Sigma, St. Louis, MO) were added for 10 min and stained with 750 µl propidium iodide in citrate buffer (30 µg/ml). Cells were analyzed by an Epics Profile-II Flow Cytometer (Coulter Corp., Miami, FL). Distribution of cells in different phases of cell cycle was calculated using *CytoLogic* software.

9. Western Blot Analysis. MDA-MB-468 cells (2 x 10^6 cells/dish) were plated in 100 mm dishes and were allowed to adhere for 24 h. Twenty four hours after treatment with

genistein, cells were harvested in PBS, centrifuged at 500 x g for 10 min and stored at -70°C until further analysis. Cells were solubulized in 300 µl of a buffer containing 50 mM Tris, 50 mM NaCl, 50 mM NaF, 0.2% SDS, 1% NP40, 2 mM EDTA, 100 µM Va_3Po_4. Total protein was determined using the Bio-Rad kit (Bio-Rad, Hercules, CA) and 30 µg was electrophoretically separated on a 10% polyacrylamide gel. The proteins were transferred to PVDF immobilon membrane. After blocking overnight with 2% non-fat dry milk, blots were incubated for 3 h with purified monoclonal mouse or polyclonal rabbit antibodies, followed by horseradish-peroxidase labeled anti-mouse/anti-rabbit secondary antibody. Protein was visualized with a chemiluminiscence based detection system.

Statistical Analysis. Statistical analyses were performed using Sigma Plot 3.0 software. Means and standard deviations were calculated for each treated group and the significant difference between the groups were determined using Student's t-test. p value <0.05 was considered significant.

B. RESULTS

Results from first year TASKS

In order to determine the interaction of phytoestrogens to ER, we studied their binding with ER, relative to unlabeled estradiol using sucrose density gradient analysis (**TASK1**). Recombinant as well as cellular ER (from MCF-7 cells) were used. The inhibition of $[^3H]E_2$ binding to cellular and recombinant ER is given in Table 1. The order of relative affinities of the phytoestrogens for ER as compared to estradiol are as follows; genistein >> quercetin > daidzein ≥ biochanin A ≥ kaempferol. The IC_{50} values of different compounds showed comparable rank order with cellular and recombinant ER. We also determined the type II estrogen binding sites (EBS) in MCF-7 and MDA-MB-468 cells according to the whole cell assay (7), (**TASK 2**). Quercetin bound to the type II EBS with high affinity, with an IC_{50} of 35 nM and 590 nM in MDA-MB-468 and MCF-7 cells, respectively (Table 2). Genistein, was less potent requiring > 2 mM concentrations to inhibit $[^3H]E_2$ binding in both the cell lines.

We then proceeded to study if there were differences in the conformational changes of ER bound to genistein or estradiol using CD studies (**TASK 3**). Recombinant ER was used in these experiments. When ER alone was analyzed, a minima spectra at 225 nm and a shoulder band at 212 nm was observed as expected. Both genistein and estradiol decreased the intensity of the CD spectra (Figure 1). The corresponding α-helix content for untreated ER, 10 µM genistein-bound ER and 10 µM estradiol-bound ER were 26.9%, 17.5% and 13.5% respectively. To further understand the differences in the structural and conformational state of ER bound to estradiol and genistein, we also conducted sucrose density gradients in the presence of these two ligands (continuation of TASK 3). $[^3H]$-E_2 and $[^{14}C]$-genistein were used to monitor the sedimentation profile of ER. A series of gradients were conducted at 1-10 nM $[^3H]$-estradiol and 1-10 µM $[^{14}C]$-genistein. Recombinant ER sedimented as a distinct peak with 1 to 10 nM concentrations of $[^3H]$-E_2, although 10 nM concentration yielded the maximal peak. In contrast, 10 µM genistein was needed to achieve a discernible ER peak. ERα and ERβ sedimented as the dimeric 7S form in the presence of estradiol. In contrast, genistein bound to ERα or ERβ sedimented as the monomeric 4S form in the sucrose gradient (Appendix B). We also

examined the sedimentation profile of a 1:1 mixture of ERα and ERβ. In this case also ER bound to [^{3}H]-E_2, sedimented as the dimeric form, while ER bound to genistein sedimented as 4S form (results not shown).

In order to determine if the conformational changes observed between genistein and E_2 bound recombinant ER also apply to ER present in breast cancer cells, we used ER from MCF-7 cells in our sucrose gradient analysis. Cellular ER sedimented as an 8S dimer with [^{3}H]-E_2. In contrast, genistein bound ER sedimented as a monomeric form, smaller than the recombinant ER monomer (Appendix B).

Results from second year TASKS

To determine if the effective binding between genistein and ER in our binding studies can lead to potential estrogenic effects for genistein, we measured the binding of genistein-ER complex to the consensus ERE in EMSA experiments (**TASK 4**). We have used both the recombinant ERα and ERβ in our experiments. An increase in the intensity of the band corresponding to the ERβ-ERE was observed in the presence of 500 nM genistein (Figure 2). In contrast there was no significant binding of Erα-ERE in the presence of different concentrations of genistein (data not shown).

We determined the effects of the phytoestrogens on DNA synthesis of MCF-7 and MDA-MB-468 cells (**TASK 5**). Cells were treated with 0, 10, 25, 50 and 100 μM phytoestrogens for 24 h. DNA synthesis was determined by [^{3}H]-thymidine incorporation assay. Effect of genistein and quercetin on DNA synthesis of MCF-7 cells is presented in Figures 3A and 3B respectively. Genistein treatment of 10 and 25 μM for 24 h caused a 27% and 39% increase in DNA synthesis of MCF-7 cells, respectively. By 72 h of treatment, all doses of genistein were growth inhibitory. Biochanin A, daidzein and kaempferol increased DNA synthesis at 10 μM concentratio n. However, quercetin did not have a stimulatory effect on DNA synthesis, and inhibited growth of these cells with an IC_{50} value of 17.3 μM. Genistein, biochanin A and kaempferol inhibited MCF-7 DNA synthesis with IC_{50} value of 37, 40 and 50 μM, respectively, whereas, daidzein was ineffective even at 100 μM concentration (Table 2).

Genistein significantly reduced the growth of MDA-MB-468 cells at all concentrations used (Appendix A) and the concentration needed to decrease the growth of ER-negative cells was 4-fold lower than that needed for the ER-positive cells. Genistein was more effective than quercetin in inhibiting the growth of ER-negative cells. Biochanin A and kaempferol exhibited similar effects on growth inhibition in ER-positive as well as ER-negative cells, where as daidzein did not have a significant effect in decreasing the growth of these breast cancer cells at the concentrations used.

We also examined whether the effect of genistein on cell proliferation in MCF-7 cells is mediated by its effects on growth regulatory genes such as c-*myc* and ODC (**TASK 6**). At all concentrations studied, genistein had no significant effect on c-*myc* mRNA levels after 24 h of treatment compared to untreated cells. However, when mRNA level of ODC was determined after treatment with 100 μM genistein for 2, 4 and 8 h, there was a decrease in ODC mRNA level at 8 h of treatment (Figure 4). Since the experiments were conducted with the addition of 4 nM estradiol, there was an induction of ODC mRNA at 4 h in the control cells.

Results from third year TASKS

We performed flow cytometric analysis of treated and untreated cells to determine the distribution of cells. Flow cytometric analysis of the untreated breast cancer group (**TASK 7**) showed a majority of the cells in the G_0/G_1 (59-63%) phase, and only a small percentage of cells in the G_2/M phase (10-18%) of the cell cycle in both MCF-7 and MDA-MB-468 cells. We observed a time and dose-dependent accumulation of the G_2/M phase cells after treatment with genistein in MDA-MB-468 cells (Appendix A). The increase in the population of G_2/M cells was associated with a corresponding decrease in the percentage of cells in G_0/G_1 phase of the cell cycle. Genistein treatment (100 μM) for 24 h caused 70% of the MDA-MB-468 cells and 42% of the MCF-7 cells to arrest at the G_2/M phase (Appendix B and Table 3 respectively). Quercetin treatment also caused G_2/M block, although to a lesser extent compared to genistein, in both the cell lines. The effects of G_2/M block by genistein and quercetin were much more prominent in MDA-MB-468 cells. Kaempferol, at 100 μM, arrested 35% of the MDA-MB-468 cells in the G_2/M phase of the cell cycle, while biochanin A and daidzein did not have significant effects on cell cycle distribution in these cells.

To further understand the genistein-activated pathway that induces cell cycle arrest, the expression of proteins that function during G_2/M phase transition was examined by Western blot analysis (**TASK 8**). Cyclin B1 levels were dramatically altered by genistein treatment. There was a biphasic response to genistein treatment, at 25 μM, there was a 70% increase in cyclin B1 level compared to control. However, a 40 and 52% lower cyclin B1 levels than control was found at 50 and 100 μM genistein. Western blot analysis of cdc2 showed no change at 10, 25, and 50 μM treatment. However, at 100 μM genistein, the upper band corresponding to the phosphorylated form of cdc2 showed a marked decrease. The β-actin levels were comparable in all lanes, showing that the observed differences are not due to altered levels of proteins (Appendix A).

Quercetin treatment showed a dose-dependent increase in cyclin B1, with 100 μM treatment resulting in a 30% increase. The level of cdc2 was unchanged after quercetin treatment. Thus even though cyclin B1 is altered by quercetin, there was a differential response of this protein to genistein treatment (Appendix A).

In MCF-7 cells, there was a 7 and 40% increase in cyclin B1 protein level at 25 and 50 μM genistein, after 24 h respectively, compared to control. At 100 μM, however, cyclin B1 was undetectable (Figure 5).

C. DISCUSSION

Our results show that genistein has stimulatory effects on DNA synthesis in MCF-7 cells up to 10 μM. Genistein binds to the recombinant ERα, however, this did not result in increased affinity to the ERE. In contrast, genistein-bound ERβ showed significant interaction with ERE. Genistein was reported to have a higher relative binding affinity to ERβ than ERα in a previous study (6). Genistein bound to the recombinant ERα and ERβ and sedimented the protein as 4S form, in contrast to estradiol, which sedimented it as 6S form. These data indicate that the dimerization potential of ER is deficient when it is bound to genistein. It is known for some time that ER functions in concert with several associated proteins to modulate gene transcription. It is likely that accessory proteins may help in the dimerization of the genistein bound ER and induce genistein mediated gene

transcription *in vivo*. Indeed, genistein has been reported to facilitate ER binding to coactivators and increase transactivation of a reporter gene. (10). However, the accessory proteins in the MCF-7 cellular extract may be limited or inefficient to induce dimerization of ER in the presence of genistein. In the case of ERβ, ERE may be providing allosteric conformational change to ERβ in the presence of genistein, thus showing increased affinity binding in our EMSA experiments. Indeed modulation of ER conformation by ERE has been reported (11). Genistein-mediated increase in DNA synthesis of MCF-7 cells did not persist at 72 h. A possible reason for this may be that prolonged treatment may cause changes in the ratio of ER versus co-activators and therefore estrogenic effects may be lost.

The other phyoestrogens, daidzein, biochanin A and kaempferol stimulated DNA synthesis of MCF-7 cells at 10 μM. However, biochanin A and kaempferol exhibited growth suppressive effects at higher concentrations with IC_{50} value of 40 and 50 μM, respectively. Quercetin had only inhibitory effects on DNA synthesis in these cells.

Our results confirm the findings of G_2/M phase arrest by genistein in MCF-7 breast cancer cells (12, 13) and extend these to MDA-MB-468 cells. Furthermore, we show that the G_2/M block is associated with disturbances in cell cycle regulation. Thus, in contrast to the classic estrogens and antiestrogens which exert their effects in the G_1 phase of the cell cycle in breast cancer cells (14), genistein and quercetin induce a G_2/M block (12, 13, 15-20). The order of potency of genistein > quercetin > kaempferol remains the same with antiproliferative effects as with the efficacy of cell cycle arrest.

Our studies link the action of genistein and quercetin with the regulation of cyclin B1 in both MCF-7 and MDA-MB-468 cells, suggesting that the growth inhibitory mechanism of these compounds may be independent of ER status. However, the mode of action of these two compounds on the regulation of cyclin B1 appears to be different in MDA-MB-468 cells. With genistein, increase in cyclin B1 was observed at lower concentration and was followed by a dramatic decrease at higher concentrations. With quercetin, G_2/M arrest is associated with an increase in cyclin B1.

In eukaryotic cells, cyclin B1 accumulates during the late S and G_2 phases and allows entry of cells into the M phase and is rapidly degraded at the end of mitosis, allowing the cells to divide (21). Inappropriate accumulation of cyclin B1 or its untimely degradation, have been reported to induce a G_2/M arrest in cells (22-26). Taxol treatment was reported to increase cyclin B1 protein in epidermoid carcinoma KB cells, parallel to mitotic arrest and programmed cell death (22). Similarly, treatment of HeLa S3 cells with X-irradiation was associated with G_2/M arrest and accelerated accumulation of cyclin B1 (23).

Alternately, decreased amount of cyclin B1 protein and G_2 arrest were reported after colcemid treatment (24) and high doses of ionizing radiation (25) in HeLa cells. In our study, lower concentrations of genistein treatment resulted in the accumulation of cyclin B1 compared to untreated cells. However, 100 μM genistein treatment resulted in a progressive decrease of cyclin B1 protein level. This result is consistent with a recent report showing a decrease in cyclin B1 at 100 μM genistein in MDA-MB-231 breast cancer cells (18). However, the effect of genistein at lower concentrations on cyclin B1 levels was not examined in this study (18). Our results suggest that accumulation of

cyclin B1 occurs early in G_2/M phase, whereas strong apoptotic signals generated at 100 μM genistein may lead to a degradation of the protein.

In contrast to genistein, 100 μM quercetin treatment for 24 h increased cyclin B1 levels compared to untreated controls in MDA-MB-468 cells, similar to the microtubule inhibitor nocodazole (27). These results suggest that the mechanism of G_2/M arrest by quercetin may follow a similar pathway as low doses of genistein. However, at higher concentrations, genistein may be interacting with other pathways to generate more potent apoptotic effects.

In mammalian cells, the levels of both mRNA and protein of cyclin B1 oscillate between the initiation and completion of mitosis. Growth inhibitory agents alter one of these parameters to deregulate the levels of these proteins, and cause cell cycle perturbations. For example, treatment of HeLa cells with camptothecin was reported to result in cyclin B1 accumulation, due to reduced rate of degradation of the protein (26). Also, irradiation of HeLa cells was reported to decrease cyclin B1 availability for G_2/M transition by delaying its mRNA synthesis during S phase or increase the degradation of the protein in the G_2/M phase, leading to cell cycle arrest (25). It is not clear if the changes seen in cyclin B1 level with genistein and quercetin treatment are due to alterations in the synthesis or degradation of the protein or mRNA.

Accumulation of cells in the G_2/M phase and apoptotic cell death were prominent features of the mechanism of action of genistein and quercetin in MDA-MB-468 cells. However, with genistein, inhibition of DNA synthesis appears not to be dependent of G_2/M arrest because 10 μM genistein was able to suppress DNA synthesis by 53% whereas G_2/M arrest was not apparent at this concentration. Effects of genistein were manifested as irreversible inhibition of DNA synthesis at 50 and 100 μM concentrations, with as little as 2 h of exposure in MDA-MB-468 cells. Interestingly, other structurally related phytoestrogens, such as biochanin A and kaempferol only had minor effects on apoptosis, even though these compounds had growth inhibitory effects with IC_{50} of ~45 μM. Thus, biochanin A and kaempferol may exert growth inhibition by molecular pathways different from that of genistein and quercetin. Genistein and quercetin appear to alter additional targets of signal transduction pathway, leading to their diverse effects on DNA synthesis, G_2/M arrest and apoptosis of MDA-MB-468 cells.

(9) CONCLUSIONS.

(i) Genistein did not significantly alter ERα and ERE binding in EMSA. In contrast, at 500 nM concentration, genistein facilitated ERβ-ERE binding. Our sucrose gradient experiments showed that genistein at 10 μM was unable to dimerize ERα or ERβ protein, a step that offers conformational advantage for binding of ER to ERE. In contrast, estradiol treatment resulted in the sedimentation of the protein in the dimeric 6S form.

(ii) Genistein, biochanin A, daidzein and kaempferol have growth stimulatory effects in ER-positive MCF-7 cells, although at higher concentrations, genistein, biochanin A and kaempferol were growth inhibitory. In contrast, quercetin had growth inhibitory effects at all concentrations studied.

(iii) Genistein and quercetin were potent growth inhibitors of ER-negative MDA-MB-468 breast cancer cells. Accumulation of cells at the G2/M phase and apoptotic cell death were prominent effects of these two compounds.

(iv) The effect of genistein on cyclin B1 protein level was biphasic in both MCF-7 and MDA-MB-468 cells, with lower concentrations increasing the level of the protein and higher concentrations decreasing it.

SO WHAT (SIGNIFICANCE).

Our results may have practical applications for the treatment of ER-negative breast cancer, as ER-positive cells acquire ER-negative phenotype that is more aggressive and resistant to antiestrogen therapy (28). Under these conditions, genistein and quercetin may be useful to inhibit breast cancer cell growth as their growth inhibitory effects are ER-independent. In this context, it is also important to note that genistein suppressed the growth of nude mice xenografts in ER-positive and -negative breast cancer cells (29). Investigations on the mechanism of action of phytoestrogens and their combination in chemo- and radiation therapy might be a fruitful approach to the design of improved breast cancer therapies.

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TABLE 1

Table 1. Concentrations of estradiol and phytoestrogens needed to displace 50% [^{3}H]-E_2 bound to cellular or human recombinant ER

Phytoestrogen	IC_{50} concentration, nM	
	MCF-7 ER	Recombinant ER
Estradiol	5	2
Genistein	900	60
Quercetin	4000	880
Daidzein	8100	600
Biochanin A	8600	920
Kaempferol	950	> 1000

IC_{50} values for the compounds were determined from the competition binding curves, as the concentration required to give 50% inhibition of specific binding of [^{3}H]- E_2 to the ER, (calculated on the basis of total receptor peak as 100%).

TABLE 2

Table 2. Concentrations of phytoestrogens needed to displace 50% [^{3}H]-E_2 bound to type-II EBS in MCF-7 and MDA-MB-468 cells

Phytoestrogen	IC_{50} concentration, nM	
	MCF-7	MDA-MB-468
Genistein	2100	2200
Quercetin	590	35
Daidzein	>10000	>10000
Biochanin A	>10000	4000
Kaempferol	>10000	> 10000

IC_{50} values for the compounds were determined from the competition binding curves (as described in methods for type-II EBS) and was calculated as the concentration required to give 50% inhibition of specific binding of [^{3}H]- E_2 to the type-II EBS.

TABLE 3

Table 3. IC_{50} values for phytoestrogens needed for cell growth inhibition

PHYTOESTROGEN	CELL LINE	
	MCF-7	MDA-MB-468
	IC_{50}, μM	
GENISTEIN	37 ± 3.8	8.8 ± 1.6
QUERCETIN	17.3 ± 2.7	18.1 ± 1.6
BIOCHANIN A	40 ± 4.1	44 ± 10.1
KAEMPFEROL	50 ± 3.9	47 ± 2.9
DAIDZEIN	>100	>100

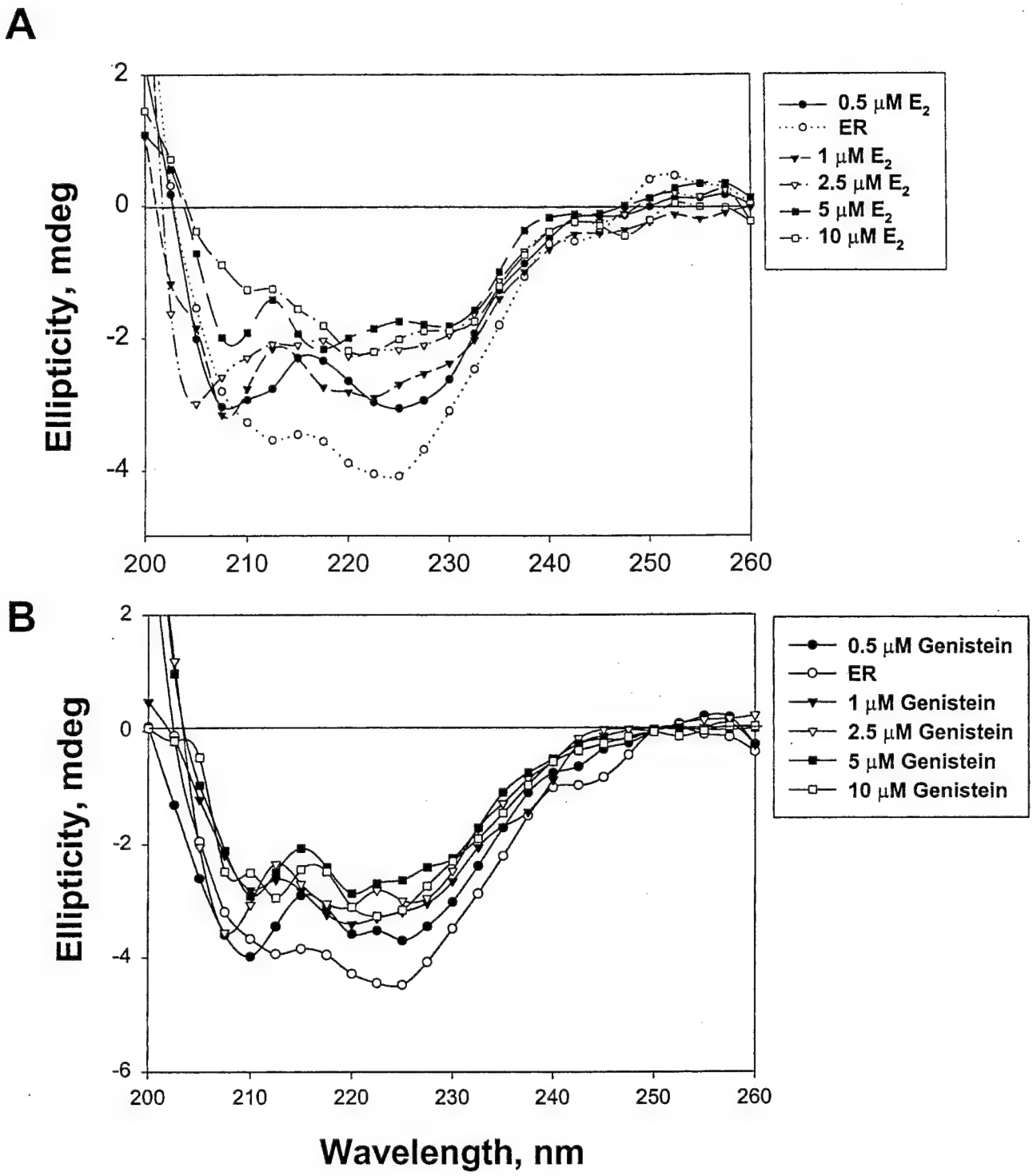
Effective concentration of phytoestrogens needed to inhibit the growth of breast cancer cells by 50% was calculated from growth curves obtained from thymidine incorporation assay, after 72 h of treatment. The values are the average of 2 separate experiments conducted in triplicate.

TABLE 4

Table 4. Phytoestrogens and nocodazole treatment on cell cycle distribution in MCF-7 cells

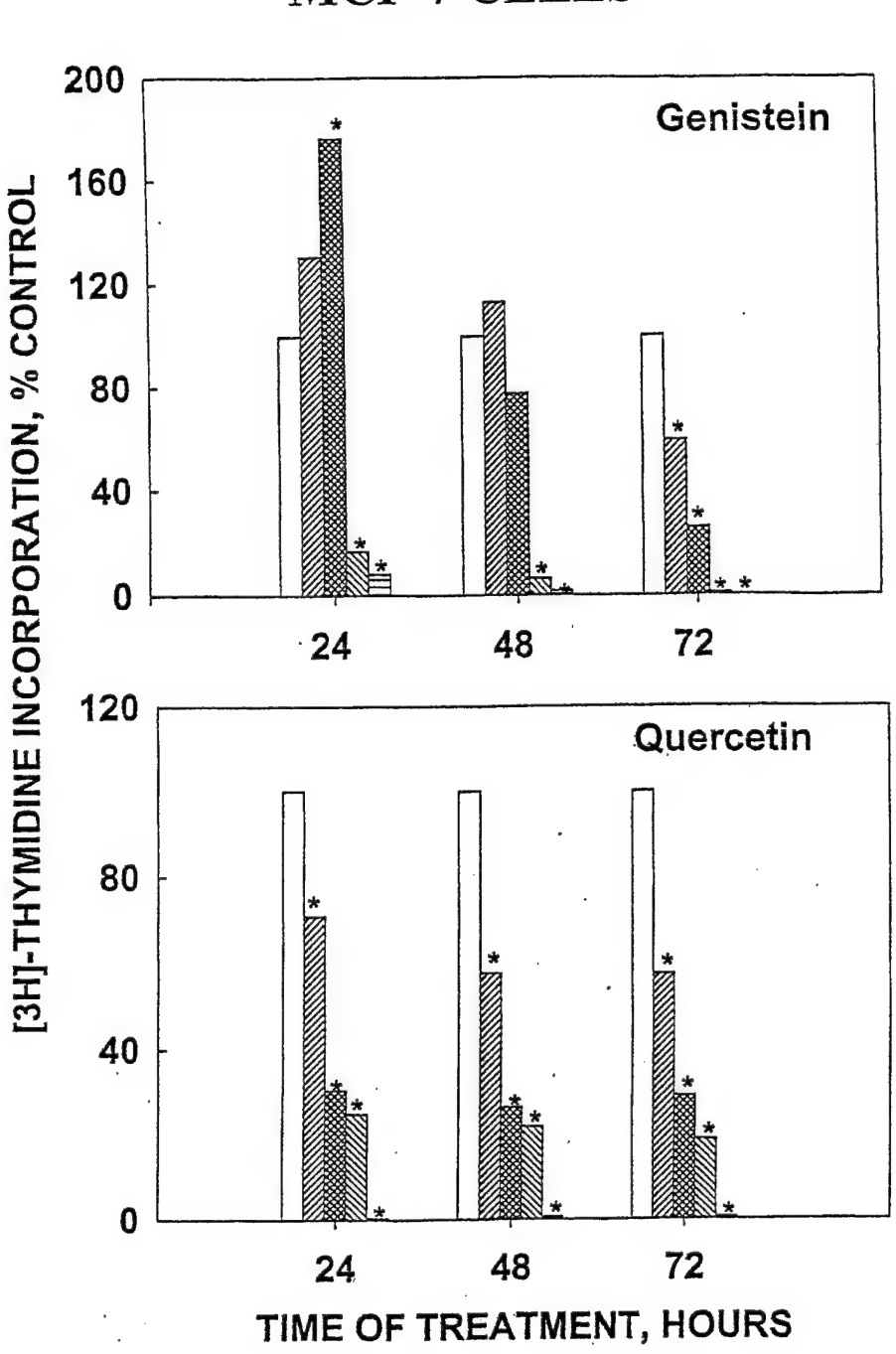
% CELLS AT 24 H AFTER TREATMENT			
TREATMENT	G0/G1	G2/M	S
GENISTEIN			
CONTROL	68.1 ± 0.7	10 ± 2.1	21.9 ± 1.4
10 μM	62.7 ± 8.2	17.2 ± 0.07	25.9 ± 0.01
25 μM	61.1 ± 0.2	13.6 ± 1.6	25.21 ± 1.6
50 μM	55.4 ± 0.2	17.6 ± 1.6	27 ± 1.4
100 μM	40.4 ± 1.2	42.4 ± 0.9	15.5 ± 1.8
QUERCETIN			
CONTROL	61.8 ± 0.8	6.03 ± 0.3	32.1 ± 0.8
10 μM	56.64 ± 1.0	9.46 ± 1.1	33.9 ± 4
25 μM	53.49 ± 2.0	22.6 ± 1.7	23.91 ± 3.6
50 μM	50.65 ± 1.8	34.55 ± 1.6	14.8 ± 0.2
100 μM	50.5 ± 1.6	37.2 ± 2.3	12.3 ± 1.5
NOCODAZOLE			
CONTROL	56.83 ± 2.6	13.6 ± 2.3	29.6 ± 3.6
0.006 μM	49.5 ± 1.9	19.4 ± 2.1	31.7 ± 0.6
0.06 μM	53.9 ± 0.7	26.9 ± 0.5	19.1 ± 0.5
0.6 μM	13.5 ± 0.4	67.4 ± 0.4	19.05 ± 0.5
6 μM	12.6 ± 1.5	72.9 ± 0.6	14.5 ± 1.6

Cell cycle distribution was determined in MCF-7 cells after treatment with genistein, quercetin and nocodazole for 24 h using Flow cytometric analysis.



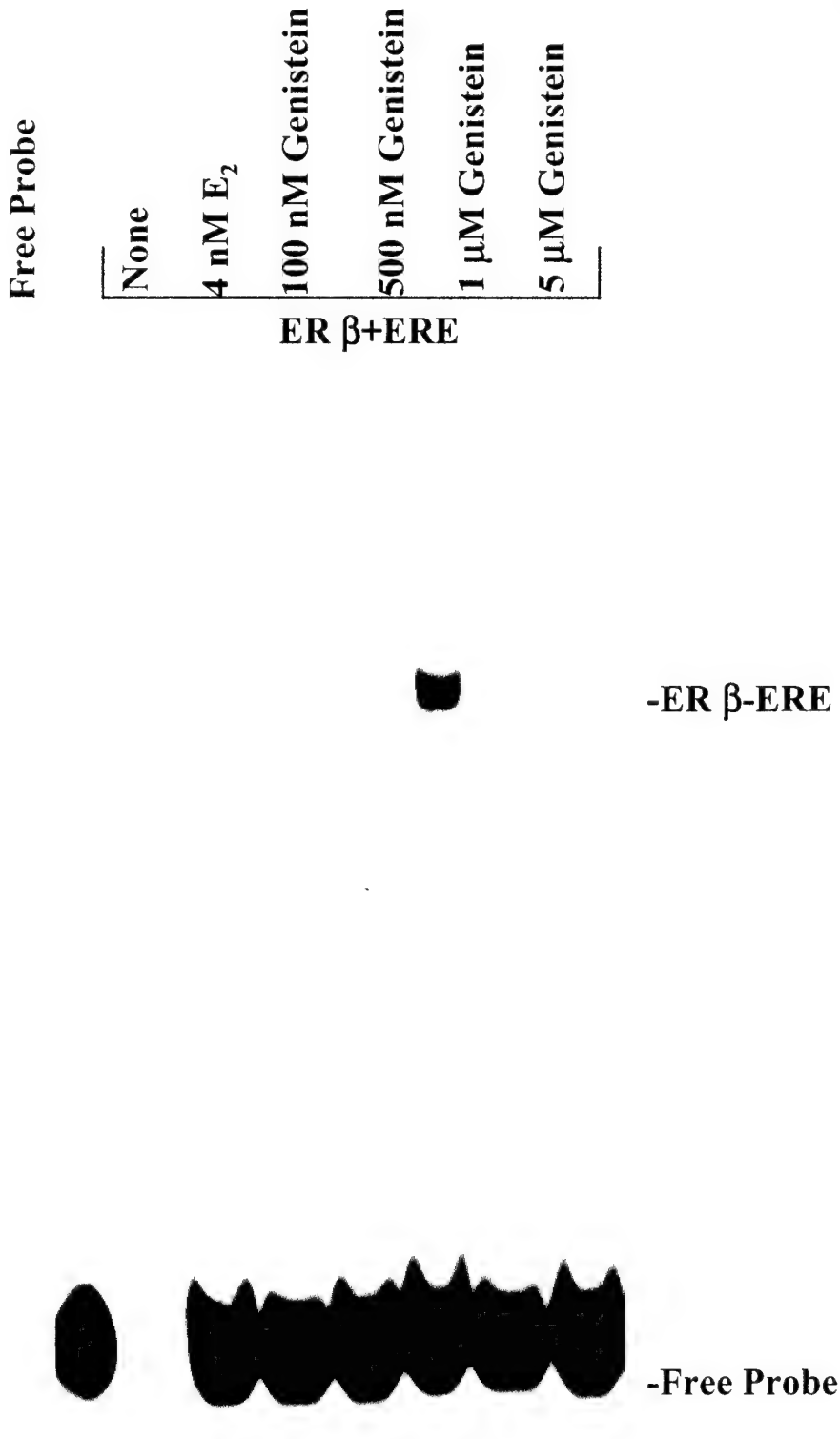
Circular dichroism spectra of human recombinant ER in the presence of increasing concentrations of (A) E_2 and (B) genistein at, 25 °C. The ER was incubated in the storage buffer overnight at room temperature. The spectra were corrected for the contribution from the buffer.

FIGURE 2

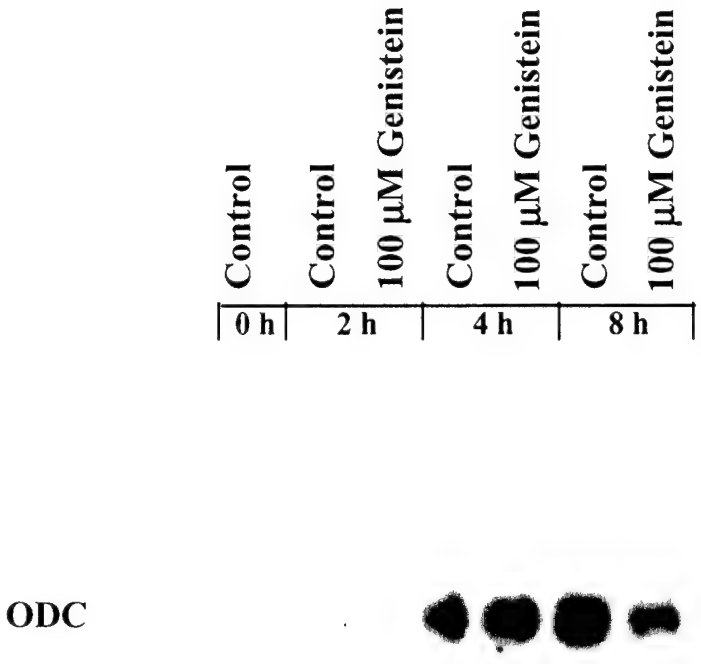


Effects of genistein and quercetin on cell proliferation. MCF-7 cells were treated with 0 (▭), 10 (▨), 25 (▩), 50 (▧) and 100 μM (▤) concentrations of (A) genistein or (B) quercetin for 24, 48 or 72 h. DNA synthesis was measured by pulse labeling for 1 h with 1 μg/ml [^{3}H]-thymidine. *Significant difference from control ($p<0.05$). Data are the mean +/- SD from two experiments.

FIGURE 3



Electrophoretic mobility shift assay to determine the ability of genistein in facilitating ER β-ERE interaction. ER β (500 ng) was incubated with 4 nM E_2 or different concentrations of genistein at 4°C for 2 h. A 4 x binding buffer (final concentration reaching 10 mm Tris-HCl, 10% glycerol, 1 mm dithiothreitol and 10 μg/ml poly(dI-dC) . poly(dI-dC)) was added for 15 min. Labeled ERE was then added to this reaction mixture and incubated for an additional 1 h at 4°C and 30 min at room temperature and loaded on to a 6% polyacylamide gel.

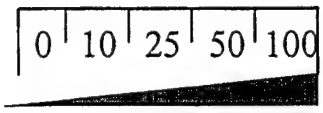


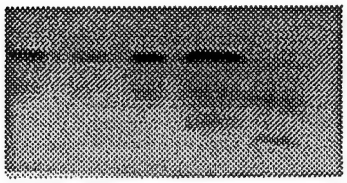
Effect of genistein on the level of ODC mRNA in MCF-7 cells. Cells were treated with 100 µM genistein for 2, 4, or 8 h. Total RNA was extracted, separated on 1% agarose gel, transferred to a nylon membrane and probed with labeled ODC cDNA.

FIGURE 5

EFFECT OF GENISTEIN AND NOCODAZOLE ON CYCLIN B1 LEVELS IN MCF-7 CELLS

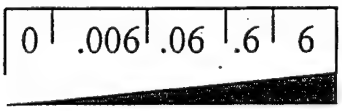
A **GENISTEIN, μM**
24 h

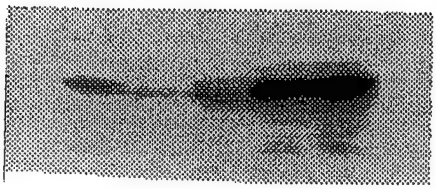




Cyclin B1

B **NOCODAZOLE, μM**
24 h





Cyclin B1

Western blots showing cyclin B1 levels in MCF-7 cells dosed with 0, 10, 25, 50, 100 μM genistein (Panel A) and 0, 0.006, 0.06, 0.6 and 6 μM nocodazole (Panel B) for 24 h.

ONCOLOGY REPORTS 7: 3-12, 2000

Effects of genistein and structurally related phytoestrogens on cell cycle kinetics and apoptosis in MDA-MB-468 human breast cancer cells

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Received October 12, 1999; Accepted October 26, 1999

Abstract. We have studied the effects of phytoestrogens (genistein, quercetin, daidzein, biochanin A and kaempferol) on proliferation, cell cycle kinetics, and apoptosis of MDA-MB-468 breast cancer cells. Genistein and quercetin inhibited cell growth with IC_{50} values of 8.8 and 18.1 μM, respectively, while the other phytoestrogens were less effective. Flow cytometric analysis showed G_2/M cell cycle arrest with 25 μM and higher concentrations of genistein. At 100 μM, genistein, quercetin and kaempferol caused accumulation of 70, 60 and 35% of cells, respectively, in G_2/M phase by 24 h. In contrast, biochanin A and daidzein were ineffective. APO-BRDU analysis revealed apoptosis with 10 μM genistein (19.5%), reaching 86% at 100 μM. Apoptosis by genistein was confirmed by Hoechst 33342 staining and fluorescence microscopy. With 100 μM quercetin, 47% of the cells were apoptotic, while the other bioflavonoids had little effect. Genistein treatment resulted in a biphasic response on cyclin B1: 70% increase in cyclin B1 level at 25 μM, and 50 and 70% decrease at 50 and 100 μM, respectively. In contrast, the action of quercetin involved an increase in cyclin B1 level. Genistein had no effect on cdc2 level up to 50 μM concentration; however, there was a decrease in the phosphorylated form of the protein at 100 μM. Quercetin had no effect on cdc2 levels. Our results suggest that the action of genistein and quercetin involves G_2/M arrest and apoptosis in MDA-MB-468 cells. Biochanin A and daidzein, although structurally related to genistein, did not share this mechanism. Thus, structurally related phytoestrogens have discrete target sites and mechanisms in their growth inhibitory action on breast cancer cells.

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Key words: genistein, phytoestrogens, apoptosis, breast cancer

Introduction

Phytoestrogens, such as genistein, quercetin, biochanin A, kaempferol and daidzein are naturally occurring phytochemicals with significant biological activities, including potential estrogenic or growth promoting, and/or anti-carcinogenic effects (1-4). The soy derived isoflavonoid genistein, was shown to have important chemotherapeutic and preventive effects in most (5-9), but not all (2,10,11), of experimental breast cancer models. In breast cancer cells, the effects of genistein are dependent on the estrogen receptor (ER) status. While low concentrations of genistein (<25 μM) are growth stimulatory in ER-positive breast cancer cells (11), it is growth suppressive at all concentrations in ER-negative cell lines (8,9). Quercetin, a flavonoid abundantly present in apples, red wine and onions, has anti-proliferative activity against several cancer cell lines including breast cancer cells (12,13) and has entered phase I clinical trials (14). Despite the general protective effect of phytoestrogens against breast cancer, their mechanism of growth inhibition and intracellular targets of action remain largely unknown.

The anti-cancer effects of several drugs are mediated by cell cycle arrest and involve modulation of the action of cyclins and cyclin dependent kinases that regulate cell cycle progression (15). Previous studies suggest G_2/M cell cycle arrest after genistein treatment (16-23). Cyclin B1 protein is the catalytic partner of cyclin dependent kinase, $p34^{cdc2}$ kinase (cdc2), that promotes microtubule spindle formation and nuclear membrane breakdown during G_2/M transition (15). Therefore, alterations in cyclin B1 and cdc2 proteins may play an important role in the effects of genistein and other phytoestrogens in breast cancer cells.

Apoptosis has been reported as critical cellular responses to a variety of chemotherapeutic agents, including genistein

(23,24). Apoptosis or programmed cell death is a genetically and metabolically controlled mechanism of cell death, and is characterized by plasma membrane blebbing, shrinkage, chromatin condensation, nuclear fragmentation and formation of membrane bound apoptotic bodies that are removed by phagocytosis (25). Furthermore, apoptosis, following treatment with a wide range of anti-cancer agents, was reported to be associated with cyclin B1 deregulation (26-32) and cdc2 dephosphorylation (33-38).

ER-negative breast cancer patients are challenged with debilitating chemotherapy and a 5-year survival rate of 53% (39). Therefore, new alternatives and additions to traditional chemotherapy are urgently needed. In this context, a better understanding of the mechanisms of action of phytoestrogens on mediators of cell cycle and apoptosis is particularly useful in that they could be used in combination with chemotherapy or radiation therapy against ER-negative tumors. Indeed, genistein and quercetin have been shown to induce radiation sensitivity in H35 hepatoma cells (40).

In the present study, we examined the effects of genistein and a group of structurally related phytoestrogens on estrogen receptor (ER)-negative MDA-MB-468 breast carcinoma cells to understand the pathways involved in phytoestrogen-mediated growth inhibition. Our results indicate G_2/M arrest and apoptosis in the action of genistein and quercetin, but these processes seem to be less important in the action of kaempferol and biochanin A.

Materials and methods

Cell culture and chemicals. MDA-MB-468 cell line was obtained from the American Type Culture Collection (ATCC, Manassas, Virginia). The cells were maintained in improved minimum essential medium (IMEM) with 10% fetal bovine serum (FBS), 4 mM glutamine, 0.4 mM sodium pyruvate, 40 µg/ml gentamycin, and 100 µg/ml each of penicillin and streptomycin.

Genistein, quercetin, daidzein, kaempferol and biochanin A were purchased from Sigma Chemical Co. (St. Louis, MO). Stock solutions were made in dimethylsulfoxide (DMSO), aliquoted, and frozen until use. The APO-BRDU™ kit was purchased from Pharmingen (San Diego, CA). The antibodies for cyclin B1, cyclin D1 and cyclin E were obtained from Neomarkers (Union City, CA). The antibody for C-terminal domain of cdc2 and phosphotyrosine antibody were purchased from Upstate Biotechnology (Lake Placid, NY).

[^{3}H]-thymidine incorporation assay. MDA-MB-468 cells ($0.5x10^6$/dish) were plated in 60 mm culture dishes in triplicate and were allowed to adhere for 24 h. Cells were dosed with phytoestrogens at 0, 10, 25, 50 and 100 µM concentrations for 24, 48 or 72 h. Control group received DMSO, equal to the volume used in drug studies (40 µl/dish). After the treatment period, the cells were pulsed with 1 µCi/ml of [^{3}H]-thymidine for 1 h. Cells were then washed twice in ice-cold PBS and two times in 5% ice-cold trichloroacetic acid and later lysed in 1 N NaOH. Lysates were transferred into vials containing liquid scintillation fluid and the radioactivity was quantitated by scintillation counting, using a Beckman Scintillation Counter, LS 5000 TD (Beckman Instruments, CA).

Reversal of cell growth inhibition. Cells were plated as described above. Groups of cultures were treated with genistein for different periods (2, 4, 6, 8, 12, 16 and 24 h), and media changed to drug-free medium. Twenty-four hours later, the cells were dosed with 1 µCi/ml of [^{3}H]-thymidine for 1 h, and the radioactivity incorporated into cellular DNA was measured by liquid scintillation counting.

Flow cytometric determination of cell cycle analysis. MDA-MB-468 cells ($2x10^6$ cells/dish) were allowed to adhere to the plate for 24 h and then dosed with genistein, or other phytoestrogens. After 24, 48 or 72 h of treatment, media was removed, 2 ml citrate buffer (40 mM citrate-trisodium, 250 mM sucrose and 5% DMSO) added and cells frozen at -70°C until further analysis. For cell cycle analysis, cells were thawed and harvested to collect the pellet. The cell pellet was treated with trypsin for 10 min, and trypsin inhibitor and RNase (Sigma, St. Louis, MO) were added for 10 min and stained with 750 µl propidium iodide in citrate buffer (30 µg/ml). Cells were analyzed by an Epics Profile-II Flow Cytometer (Beckman Coulter Inc., Fullerton, CA). Distribution of cells in different phases of cell cycle was calculated using *CytoLogic* software.

Western blot analysis. Cells were plated as described above for flow cytometry. Twenty-four hours after treatment with genistein, cells were harvested in PBS, centrifuged at 500 x g for 10 min and stored at -70°C until further analysis. Floating cells were also harvested and added to the adherent cells in each treatment group. Cells were solubulized in 300 µl of a buffer containing 50 mM Tris, 50 mM NaCl, 50 mM NaF, 0.2% SDS, 1% NP-40, 2 mM EDTA and 100 µM Na_3PO_4. Total protein was determined using the Bio-Rad kit (Bio-Rad, Hercules, CA) and 30 µg was electrophoretically separated on a 10% polyacrylamide gel. The proteins were transferred to PVDF immobilon membrane. After blocking overnight with 2% non-fat dry milk, blots were incubated for 3 h with purified monoclonal mouse or polyclonal rabbit antibodies, followed by horseradish-peroxidase labeled anti-mouse/anti-rabbit secondary antibody. Protein was visualized with a chemiluminescence based detection system.

APO-BRDU labeling studies. MDA-MB-468 cells ($2x10^6$/dish) were plated and dosed with genistein for 24 h. Floating and adherent cells were harvested in PBS and fixed in 1% paraformaldehyde. After two washings with PBS, the cell pellet was fixed in 70% ethanol and frozen at -20°C until further use. Apoptosis was quantified using the APO-BRDU kit with minor modifications to the manufacturer's instructions. Briefly, cell pellet was incubated with bromolated deoxyuridine triphosphate (BRDU) for 24 h in a 28°C water bath. After the incubation period, cells were treated with fluorescein-labeled anti-BRDU monoclonal antibody for 1 h, stained with propidium iodide/RNase solution for 30 min in dark, and analyzed by flow cytometry.

Analysis of apoptosis by Hoechst 33342 dye staining. Cells ($3.5x10^4$/dish) were plated in 4-well LabTek chambered cover glass plates (Nalge Nunc Int., IL). After allowing for adherence of cells for 48 h, different concentrations of genistein were

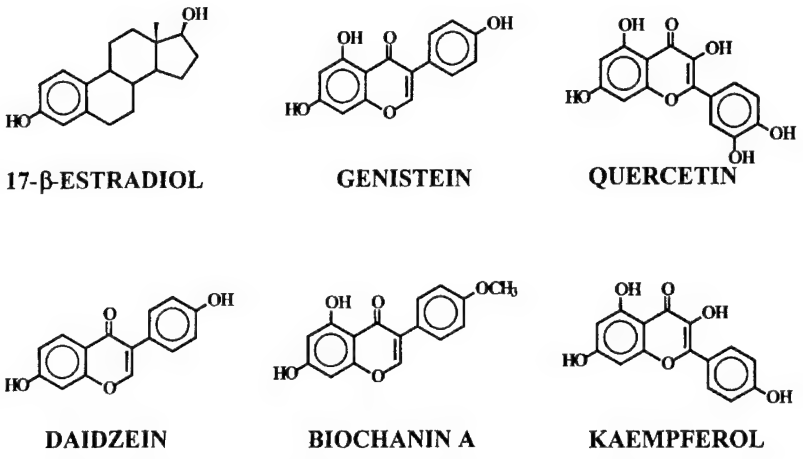

Figure 1. Chemical structures of 17-ß-estradiol and phytoestrogens used in this study.

added to the plates. After 24 h treatment, cells were fixed in 4% paraformaldehyde with 4 µg/ml Hoechst 33342 dye and incubated at 37°C for 30 min. Stained nuclei were observed under Zeiss ICM 405 inverted microscope (magnification x1000) using a UV filter in the range of 395-450 nm. Apoptotic cells, characterized by nuclear shrinkage and fragmentation, were counted from 3 random fields with at least 100 cells per field.

Statistical analysis. Statistical analyses were performed using Jandel Scientific SigmaStat software. Means and standard deviations were calculated for each treated group and the significant difference between the groups were determined using Student's t-test. p-value <0.05 was considered statistically significant.

Results

Growth inhibitory effects of phytoestrogens. The chemical structure of phytoestrogens used in this study are shown in Fig. 1. In order to determine the effects of phytoestrogens on cell proliferation, MDA-MB-468 cells were treated with 0, 10, 25, 50 and 100 µM concentrations of genistein, quercetin, kaempferol, biochanin A and daidzein. After 24, 48 or 72 h of treatment, cells were pulsed with [^{3}H]-thymidine for 1 h to measure the level of DNA synthesis. Fig. 2 show the effects of genistein and quercetin treatment on [^{3}H]-thymidine incorporation of MDA-MB-468 cells. Twenty-four hour treatment with 10 µM genistein or quercetin significantly reduced [^{3}H]-thymidine incorporation by 47 and 32%, compared to the control, respectively ($p<0.05$, n=6). At 100 µM, genistein suppressed DNA synthesis by 90%, while 70% reduction was found with quercetin. To quantify the effects of these and other phytoestrogens, we determined the IC_{50} values, the mean concentration of phytoestrogens required for 50% growth inhibition (Table I). Among the phytoestrogens studied, genistein was the most potent growth inhibitor with an IC_{50} value of 8.8±1.6 µM, followed by quercetin (IC_{50} = 18.1±1.6 µM). Genistein exhibited a 5-fold higher potency than its methoxy derivative, biochanin A. Kaempferol, which differs from quercetin by lacking an -OH group on the 5' position of its B ring, is 2.6-fold less effective than quercetin. Interestingly, a major phytoestrogen in soy, daidzein did not inhibit the growth of MDA-MB-468 cells even at 100 µM concentration.

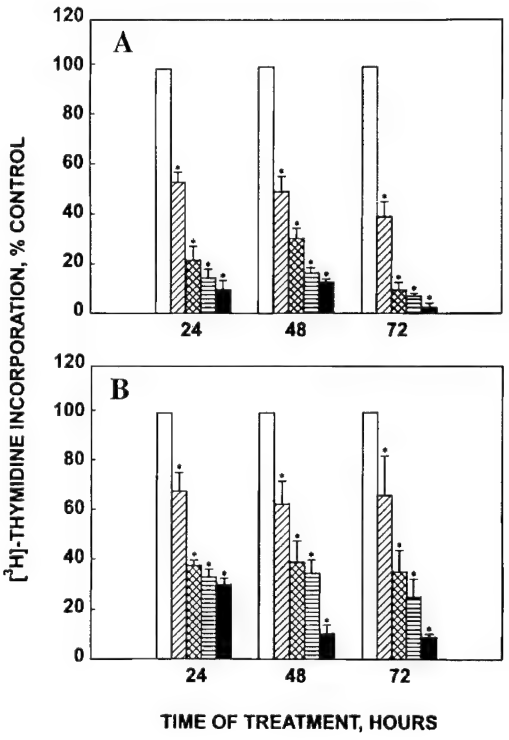

Figure 2. Effects of genistein and quercetin on cell proliferation. MDA-MB-468 cells were treated with 0 (white bar), 10 (hatched bar), 25 (double hatched bar), 50 (striped bar) and 100 µM (black bar) concentrations of: (A), genistein or (B), quercetin for 24, 48 or 72 h. DNA synthesis was measured by pulse labeling for 1 h with 1 µg/ml [^{3}H]-thymidine. *Significant difference from control ($p<0.05$). Data are the mean ± SD from two experiments.

Effect of phytoestrogens on cell cycle kinetics. To assess the effects of phytoestrogens on cell cycle, cells were treated

Table I. IC_{50} values for phytoestrogens needed for cell growth inhibition.

Phytoestrogen	IC_{50}, μM (concentration ± SD)
Genistein	8.8±1.6
Quercetin	18.1±1.6
Biochanin A	44.0±10.1
Kaempferol	47.0±2.9
Daidzein	>100

MDA-MB-468 cells were treated with each of the phytoestrogens at 0, 10, 25, 50 and 100 μM concentrations for 24, 48 or 72 h. Cell growth was determined by [^{3}H]-thymidine incorporation assay. IC_{50} value was calculated from the growth curves as the concentration that inhibited 50% of cell growth. The values are an average of two separate experiments conducted in triplicate.

with these compounds (0-100 μM) for 24, 48 or 72 h, and stained with propidium iodide. Flow cytometry profiles of cells treated for 24 h with genistein, quercetin and kaempferol are shown in Fig. 3. Genistein and quercetin induced cell cycle arrest by 24 h of treatment. Interestingly, arrest of cells in G_2/M phase reached significant levels only at 25 μM concentration, although inhibition of DNA synthesis was seen at 10 μM genistein. At 100 μM genistein, 70% of the cells were found in G_2/M phase of the cell cycle. The G_2/M arrest was associated with a corresponding decrease in the percentage of cells in the G_0/G_1 phase of the cell cycle. The percentage of cells in each phase of the cell cycle after 24 h of treatment with phytoestrogens are shown in Table II. Quercetin treatment at 50 and 100 μM concentrations showed an accumulation of cells in G_2/M phase leading to 41 and 60% by 24 h, respectively. Kaempferol was less effective than quercetin in inducing a G_2/M arrest. In contrast, biochanin A and daidzein did not have significant effects on cell cycle progression. Similar results were obtained after 48 and 72 h of treatment. Treatment of cells with 100 μM genistein, quercetin, kaempferol, daidzein or biochanin A for 72 h resulted in G_2/M phase accumulation of cells corresponding to 87, 67, 35, 21 and 9%, respectively. These results show that inhibition of DNA synthesis occurred with genistein and quercetin, at lower concentration than those required for G_2/M arrest.

Effects of genistein on reversal of cell growth. We next examined if genistein-induced inhibition of DNA synthesis is reversible. If inhibition of DNA synthesis results from moderate changes in the level of certain mediators without irrepairable damage to DNA, recovery of the cells can be expected when the damaging agent is removed.

Cells were treated with genistein for defined time periods, and then the drug was removed and cells treated with drug-free medium. Genistein treatment at 50 and 100 μM caused irreversible inhibition of DNA synthesis ($p<0.05$) even after 2 h of exposure (Fig. 4A). After 8 h, genistein treatment with 25 μM concentration was cytotoxic to these cells, with growth inhibition comparable to cells which had continuous exposure to the drug (Fig. 4B). Furthermore, genistein induced irreversible changes in DNA synthesis of MDA-MB-468 cells by 16 h even at 10 μM concentration. Thus, inhibition of DNA synthesis observed after 24 h treatment with 10 μM

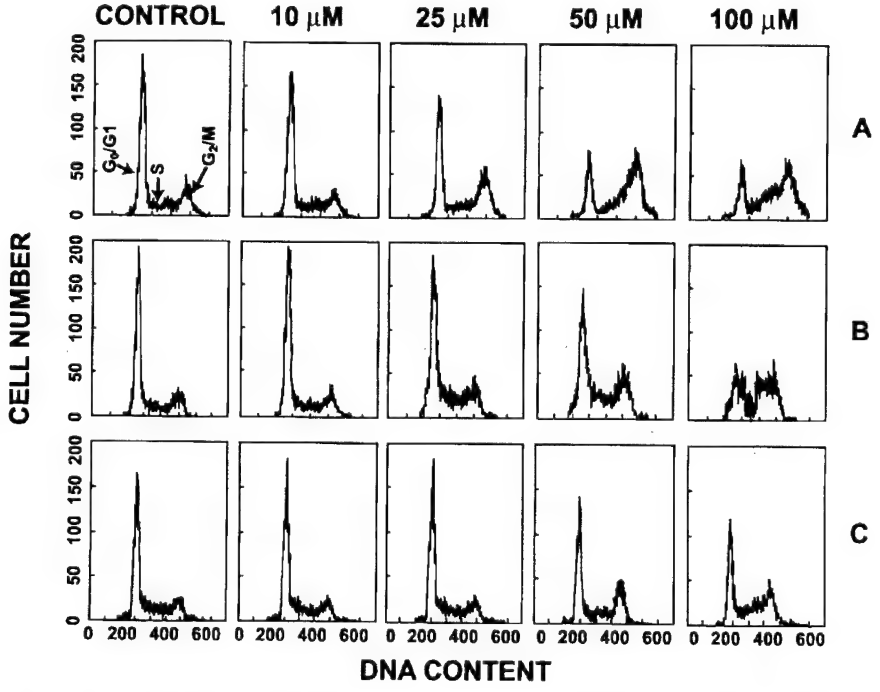

Figure 3. Flow cytometry profile of cell cycle distribution of MDA-MB-468 cells. Cells were treated with 0, 10, 25, 50 and 100 μM concentrations of: (A), genistein; (B), quercetin or (C), kaempferol for 24 h. RNase treated and propidium iodide stained cells were sorted by flow cytometry.

Table II. Effect of phytoestrogens on cell cycle distribution of MDA-MB-468 cells.

Phyto-estrogen	Concentration (μM)	G_0/G_1	S	G_2/M
Control	0	59.0±1.7	22.4±1.1	17.5±1.1
Genistein	10	65.5±1.6[a]	18.3±1.6	16.2±1.9
	25	47.9±0.9[a]	23.3±1.3	33.2±0.7[a]
	50	27.7±0.3[a]	17.0±0.9	49.5±0.7[a]
	100	27.3±3.2[a]	3.0±0.9[a]	70.0±4.3[a]
Quercetin	10	52.1±2.1[a]	32.6±4.3[a]	15.2±2.5
	25	50.5±0.7[a]	27.7±1.2	21.7±0.7
	50	48.4±1.3[a]	24.7±2.5	41.2±2.2[a]
	100	35.0±5.1[a]	5.0±2.1[a]	60.0±5.2[a]
Kaempferol	10	58.8±0.2	20.9±0.9	20.3±1.1
	25	58.2±0.5	20.6±1.3	21.2±1.7
	50	46.4±4.2[a]	19.5±0.9	34.1±3.3[a]
	100	45.6±2.7[a]	19.2±1.1	35.1±1.7[a]
Biochanin A	10	59.7±0.4	22.9±1.1	17.4±0.8
	25	60.9±0.1	20.7±1.1	18.3±1.2
	50	60.3±1.2	20.9±1.0	18.7±0.5
	100	59.7±1.6	20.9±0.1	19.3±1.5
Daidzein	10	57.1±1.8	19.5±1.1	20.0±2.7
	25	52.6±1.2	20.7±1.1	23.6±2.5
	50	53.8±5.7	19.1±0.7	22.8±5.4
	100	52.2±5.7	22.5±3.9	21.8±2.3

Flow cytometric analysis was performed and the percentage of cells in the G_0/G_1, S and G_2/M phases of the cell cycle was calculated using the *CytoLogic* software, after treatment of MDA-MB-468 cells with different phytoestrogens for 24 h. [a]Significantly different from control, $p<0.05$ (n=6).

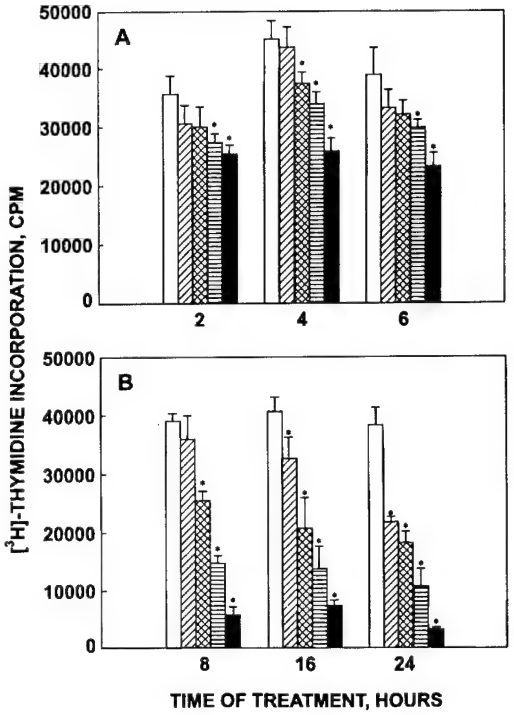

Figure 4. Growth reversal of MDA-MB-468 cells after treatment with genistein. Cells were treated with increasing concentrations of genistein for: (A), 2, 4 and 6 h and (B), 8, 16 and 24 h. After treatment, media was replaced with fresh media without drug for an additional 24 h and [^{3}H]-thymidine incorporation assay was performed as described in Materials and methods. *Significant difference from control ($p<0.05$). The experiment was performed in triplicate. Error bars indicate SD.

genistein may be the result of irreversible damage to DNA or other critical components of cell cycle machinery.

Effects of phytoestrogens on apoptotic cell death. MDA-MB-468 cells were treated with phytoestrogens for 24 h and the percentage of apoptotic cells was assessed by the APO-BRDU kit (Pharmingen, CA). In this assay, induction of apoptosis is detected by an increase in DNA fragments that are labeled with BRDU and a fluorescent tagged antibody using flow cytometry. Fig. 5 shows a representative view of the cytograms (Fig. 5A) and histograms (Fig. 5B) obtained after treatment of cells with increasing concentrations of genistein. Treatment of cells with 10, 25, 50 and 100 μM genistein resulted in significant increase in apoptotic cells, 19, 34, 64 and 86%, respectively, compared to 1.6% in the control cells ($p<0.01$, n=4). The effect of phytoestrogen treatment on the percentage of apoptotic cells is presented in Table III. Quercetin exposure at 100 μM resulted in 47% apoptotic cells, whereas biochanin A treatment caused 11% apoptosis. In contrast, there was no significant level of apoptosis by treatment with kaempferol and daidzein.

To confirm genistein-induced apoptosis, cells were treated with genistein for 24 h and stained with Hoechst 33342. Stained nuclei were then visualized under a fluorescence microscope. A representative view of dye-stained nuclei is presented in Fig. 6. Nuclear condensation and apoptosis was observed even at 10-25 μM concentrations of genistein. Quantification of apoptotic cells by counting non-apoptotic and apoptotic cells yielded values comparable to that of APO-BRDU analysis at 10-25 μM genistein. At 50-100 μM genistein, percentage of apoptotic cells counted after Hoechst 33342 staining were not as quantitative as those from APO-BRDU assay, a possible reason being the loss of apoptotic cells during staining and washing.

Effects of genistein and quercetin on the expression of cell cycle regulatory proteins. To further understand the genistein-activated pathway that induces cell cycle arrest and apoptosis, the expression of proteins that function during G_2/M phase transition was examined by Western blot analysis. Fig. 7 shows the effect of genistein on the expression of cyclin B1 and cdc2. ß-actin was used as a control to assess differences in

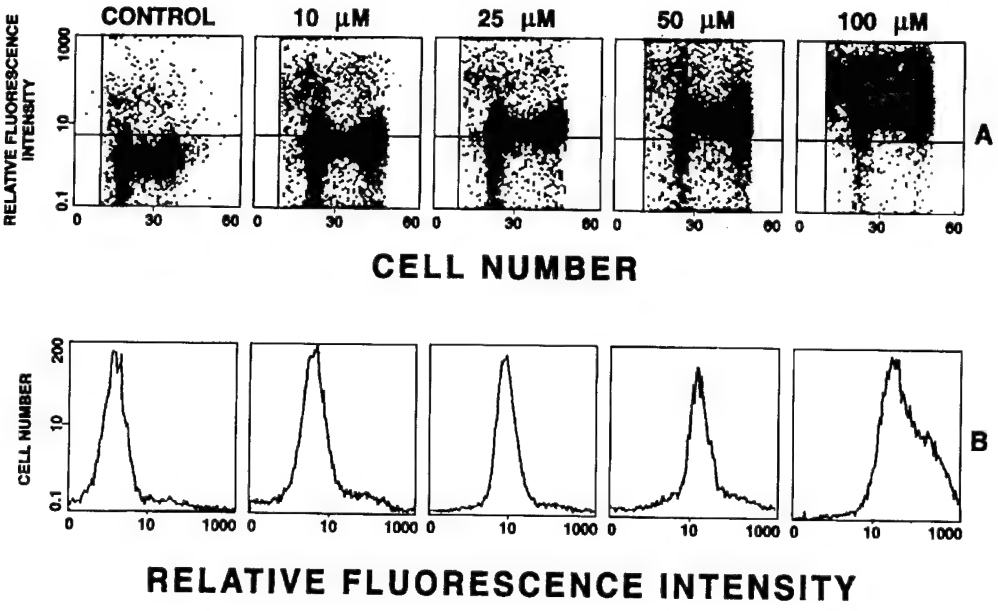

Figure 5. Genistein-induced apoptotic cell death of MDA-MB-468 cells. Cells were treated with 0, 10, 25, 50 and 100 µM concentrations of genistein for 24 h. Harvested cells were stained with both propidium iodide and fluorescein-BRDU monoclonal antibody and were analyzed by flow cytometry. (A), Cytograms with cell number on x-axis vs. relative fluorescence intensity (log green fluorescein BRDU) on y-axis. (B), Histograms showing percentage of apoptotic cells. x-axis, fluorescence intensity; y-axis, cell number. Similar results were obtained in two separate experiments.

Table III. Effect of phytoestrogens on percentage apoptosis of MDA-MB-468 cells.

	% apoptosis by 24 h treatment with:				
Phytoestrogen	0 µM	10 µM	25 µM	50 µM	100 µM
Genistein	1.6±0.6	19.5±9.8[a]	34.7±8.3[a]	64.3±15.0[a]	86.0±4.0[a]
Quercetin	1.6±0.6	3.3±0.4	12.3±1.5[a]	18.1±5.6[a]	47.2±1.5[a]
Biochanin A	1.5±0.6	1.5±0.1	3.8±1.3	6.1±0.3[a]	11.2±1.2[a]
Daidzein	1.5±0.8	1.3±0.1	2.4±0.1	2.9±0.1	1.7±0.6
Kaempferol	1.9±0.1	2.5±1.7	3.4±0.7	3.7±0.7	4.8±0.4[a]

APO-BRDU analysis for apoptosis was determined after treatment of MDA-MB-468 cells with different phytoestrogens for 24 h. DNA fragments in apoptotic cells are end-labeled with BRDU and incubated with fluorescent tagged BRDU-specific antibody. Fluorescence intensity generated is proportional to the percentage apoptosis as determined by flow cytometry. [a]Significantly different from control, $p<0.01$ (n=6).

the amount of protein loading and transfer during the Western analysis. Cyclin B1 levels were dramatically altered by genistein treatment. There was a biphasic response with a 70% increase in cyclin B1 level at 25 µM genistein, compared to the control. In contrast, at 50 and 100 µM genistein, cyclin B1 level was reduced to 50 and 30% of the control, respectively. This biphasic response of cyclin B1 may represent the ability of genistein to act at multiple levels of gene expression such as transcription, mRNA stability, and/or protein synthesis/degradation (41).

Western blot analysis of cdc2 showed no change at 10, 25 and 50 µM genistein treatment. However, at 100 µM genistein, the upper band corresponding to the phosphorylated form of cdc2 showed a marked decrease. This decrease corresponded with a decrease in total phosphorylation level also, as determined from Western blot analysis using phosphotyrosine antibody (not shown). In contrast, ß-actin levels were not altered.

We also examined the effect of genistein on cyclin D1 and cyclin E, the G1 cyclins known to mediate the progression of cells through G_1 to S phase. The Western blot analysis using anti-cyclin D1 antibody and anti-cyclin E showed that genistein at 10-100 µM concentrations did not have significant effects on these cyclins (results not shown).

Fig. 8 shows the effect of quercetin on cyclin B1 and cdc2. Quercetin treatment showed an increase in cyclin B1, with

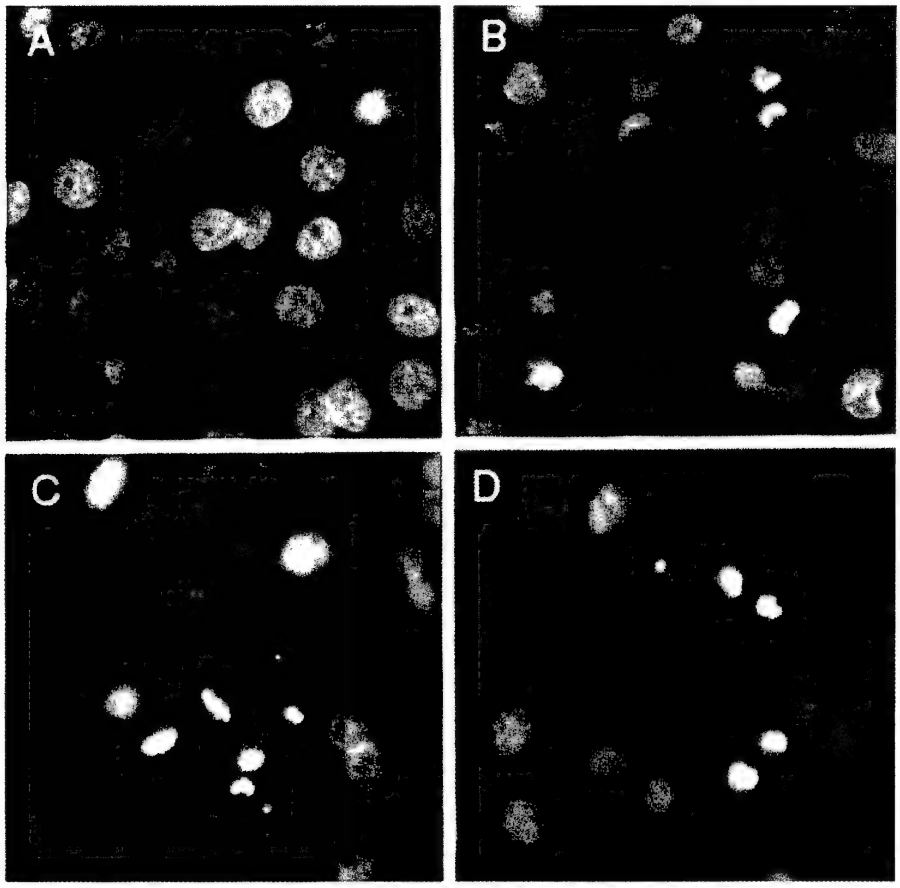

Figure 6. Genistein induced apoptotic cell death of MDA-MB-468 cells as determined by Hoechst 33342 staining. Cells were treated with genistein [0, 10, 25 and 50 μM, (A-D), respectively] for 24 h and fixed in 4% paraformaldehyde. Then the cells were treated with Hoechst 33342 dye and the stained nuclei were visualized under fluorescence microscope. Irregular and condensed or fragmented nuclei were observed as characteristic features of apoptosis. To quantify apoptosis, cells from three different fields were counted, with about 100 cells per field.

100 μM treatment resulting in a 30% increase. Even though 100 μM quercetin caused 47% of the cells to undergo apoptosis, unlike genistein, quercetin did not cause a decrease in cyclin B1. Furthermore, the level of cdc2 was unchanged after quercetin treatment. Thus genistein and quercetin appear to have different modes of action on cyclin B1 synthesis and/or degradation although both agents caused a significant level of apoptosis in MDA-MB-468 cells.

Discussion

Our results show that genistein and quercetin are highly efficient in inhibiting the growth of MDA-MB-468 cells. Accumulation of cells in the G_2/M phase and apoptotic cell death were prominent features of the mechanism of action of both compounds. However, with genistein, inhibition of DNA synthesis appears not to be dependent of G_2/M arrest because 10 μM genistein was able to suppress DNA synthesis by 53% whereas G_2/M arrest was not apparent at this concentration. Both genistein and quercetin altered cyclin B1 levels. With genistein, an increase in cyclin B1 was observed at 25 μM concentration and was followed by a dramatic decrease at higher concentrations. With quercetin, G_2/M arrest and apoptosis are associated with an increase in cyclin B1 protein level. Interestingly, other structurally related phytoestrogens, such as biochanin A and kaempferol only had minor effects on apoptosis, even though these compounds had growth inhibitory effects with IC_{50} of ~45 μM. Thus, biochanin A and kaempferol may exert growth inhibition by molecular pathways different from that of genistein and quercetin.

Our experiments provide new insight into the structure-function relationships on the growth inhibitory effects of phytoestrogens. All phytoestrogens showing growth inhibitory effects have a 5'-OH group on the A ring (Fig. 1). Therefore, this group might be involved in the anti-proliferative effects of phytoestrogens. In contrast, daidzein which is structurally similar to genistein in all respects except lacking the 5'-OH group on the A ring, is ineffective in inducing growth inhibition, cell cycle arrest or apoptosis. Furthermore, the 4'-OH group on B ring of genistein seems to be important in eliciting growth inhibitory effects, since these effects are greatly reduced when the hydroxyl group is methylated (biochanin A). When the phenolic B ring is attached to the 2 position of pyran C ring as in genistein, instead of 3 position (quercetin or kaempferol), the potency of anti-proliferative effects seem to be maximal. Quercetin with an additional 5'-OH group on B ring compared to kaempferol is a better anti-proliferative agent than kaempferol in our studies.

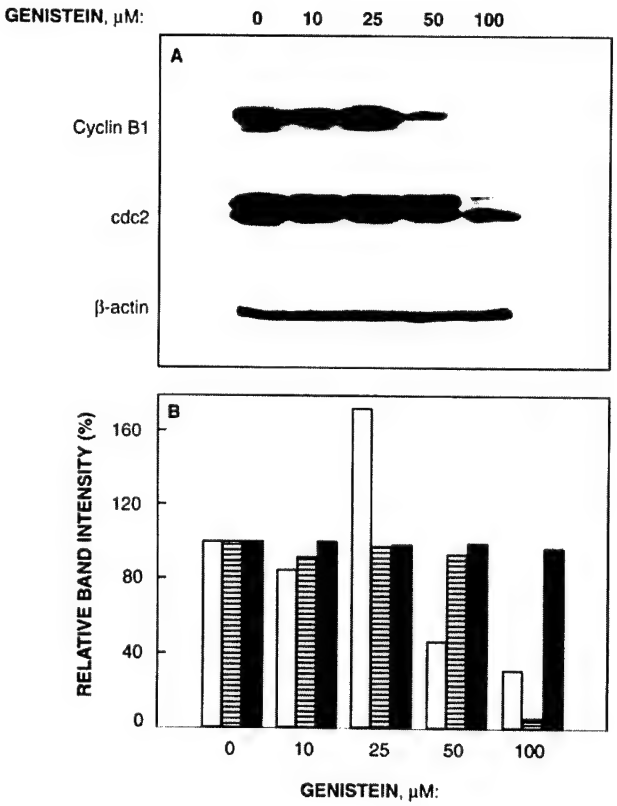

Figure 7. Western blot analysis of cyclin B1, cdc2 and ß-actin expression in MDA-MB-468 cells treated with 0, 10, 25, 50 and 100 μM of genistein for 24 h. Protein lysates were electrophoresed, transferred onto a PVDF immobilon membrane and treated with antibodies against cyclin B1, cdc2 or ß-actin. Similar results were obtained in three independent experiments. Variations in intensity were less than 10%.

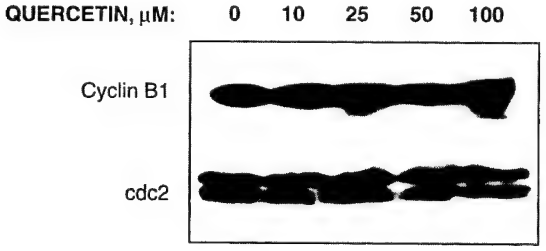

Figure 8. Western blot analysis of cyclin B1 and cdc2 protein expression in MDA-MB-468 cells treated with 0, 10, 25, 50 and 100 μM concentrations of quercetin for 24 h. Protein lysates were electrophoresed, transferred onto a PVDF immobilon membrane and probed with an antibody against cyclin B1 or cdc2. Similar results were obtained in two separate experiments.

Growth inhibition by genistein, quercetin and kaempferol was associated with increased accumulation of G_2/M phase cells in our studies. While kaempferol and biochanin A showed growth inhibition with IC_{50} value in a similar range kaempferol had more significant effects on cell cycle arrest than biochanin A. The order of potency of genistein > quercetin > kaempferol remains the same with anti-proliferative effects as with the efficacy of cell cycle arrest. Our results extend the findings of G_2/M phase arrest by genistein in MCF-7 (19,20) and MDA-MB-231 cells (21-23) to MDA-MB-468 breast cancer cells and further link this blockade to dysregulation of cell cycle associated genes and apoptosis. Thus, in contrast to the classic estrogens and anti-estrogens which exert their effects in the G_1 phase of the cell cycle (42), genistein and quercetin induce a G_2/M block (13,19-23).

Cell cycle arrest by genistein and quercetin suggests that these phytoestrogens can interfere with the synthesis or degradation of cyclins and cyclin dependent kinases (CDKs) or their inhibitors. In eukaryotic cells, cyclin B1 accumulates during the late S and G_2 phases, and allows entry of cells into the M phase and is rapidly degraded at the end of mitosis, allowing the cells to divide (43). Inappropriate accumulation of cyclin B1 or its untimely degradation have been reported to induce a G_2/M arrest in cells (26,30-32). Taxol treatment was reported to increase cyclin B1 protein in epidermoid carcinoma KB cells, parallel to mitotic arrest and programmed cell death (30). Similarly, treatment of HeLa S3 cells with X-irradiation was associated with G_2/M arrest and accelerated accumulation of cyclin B1 (32).

Alternately, decreased amount of cyclin B1 protein and G_2 arrest were reported after colcemid treatment (26) and high doses of ionizing radiation (31) in HeLa cells. In our study, 25 μM genistein treatment resulted in the accumulation of cyclin B1 compared to untreated cells. However, 50 and 100 μM genistein treatment resulted in a progressive decrease of cyclin B1 protein level. This result is consistent with a recent report showing a decrease in cyclin B1 at 100 μM genistein in MDA-MB-231 breast cancer cells (22). However, the effect of genistein at lower concentrations on cyclin B1 levels was not examined in this study (22). Our results suggest that accumulation of cyclin B1 occurs early in G_2/M phase, whereas strong apoptotic signals generated at 50 and 100 μM genistein may lead to a degradation of the protein.

In contrast to genistein, 100 μM quercetin treatment for 24 h increased cyclin B1 levels compared to untreated controls, similar to the microtubule inhibitor nocodazole (44). These results suggest that the mechanism of G_2/M arrest by quercetin may follow a similar pathway as low doses of genistein. However, at higher concentrations, genistein may be interacting with other pathways to generate more potent apoptotic effects. In this regard, it is important to note that percentage of apoptotic cells with 100 μM quercetin was similar to that attained with 25 μM genistein, and cyclin B1 levels were higher at these concentrations in both cases.

In mammalian cells, the levels of both mRNA and protein of cyclin B1 oscillate between the initiation and completion of mitosis. Growth inhibitory agents alter one of these parameters to deregulate the levels of these proteins, and cause cell cycle perturbations. For example, treatment of HeLa cells with camptothecin was reported to result in cyclin B1 accumulation, due to reduced rate of degradation of the protein (29). Also, irradiation of HeLa cells was reported to decrease cyclin B1 availability for G_2/M transition by delaying its mRNA synthesis during S phase or increase the degradation of the protein in the G_2/M phase, leading to cell cycle arrest

(31). It is not clear if the changes seen in cyclin B1 level with genistein and quercetin treatment are due to alterations in the synthesis or degradation of the protein or mRNA.

Premature dephosphorylation of cdc2-tyr15, has been implicated in apoptotic cancer cell death induced by chemotherapeutic agents, such as GL331 (33), 7-hydroxystaurospaurine (UCN-01) (34), caffeine (36) and taxol (37). It has been suggested that cdc2 elicits a protein phosphorylation cascade that could lead to aberrant mitotic mechanisms that play an important part in apoptotic morphology (33). In our studies, no change in the cdc2 protein level was observed with genistein treatment at 10, 25 and 50 μM concentrations. However, at 100 μM genistein, the phosphorylated form of the protein was drastically reduced. Use of phosphorylated tyrosine kinase antibodies also showed similar results in that a decrease in the level of phosphorylation was observed with 100 μM genistein (results not shown). Thus, the role of genistein as an inhibitor of tyrosine phosphorylation plays a part in its mechanism of action only at the highest concentrations examined. This lack of tyrosine kinase inhibition with low concentrations of genistein is consistent with the findings of Peterson and Barnes (45). In contrast to genistein, quercetin treatment did not have an effect on cdc2 protein in MDA-MB-468 cells.

Quercetin is known to inhibit cdc2 kinase (46), thereby exerting growth inhibitory effects. In addition, quercetin was shown to down-regulate mutant p53 protein in MDA-MB-468 cells, thus leading to significant growth inhibition (13). Genistein has been reported to increase the expression of the CDK inhibitor, $p21^{WAF1/CIP1}$ at the mRNA and protein levels (47) even in a p53 negative cell line (22), thus inhibiting cell growth through a p21-dependent pathway.

Our results show that cyclin B1 is altered by genistein and quercetin, although it is not clear at present whether it is a primary or secondary event. Effects of genistein were manifested as irreversible inhibition of DNA synthesis at 50 and 100 μM concentrations, with as little as 2 h of exposure. Other structurally related phytoestrogens, biochanin A and kaempferol are less effective in mediating the growth inhibition of these cells. Genistein and quercetin appear to alter additional targets of signal transduction pathway, leading to their diverse effects on DNA synthesis, G_2/M arrest, and apoptosis.

Our results may have practical applications for the development of phytoestrogens as therapeutic agent for ER-negative breast cancer, as ER-positive cells acquire ER-negative phenotype that is more aggressive and resistant to anti-estrogen therapy (48). Under these conditions, genistein and quercetin may be useful to inhibit breast cancer cell growth as their growth inhibitory effects are ER-independent. In this context, it is also important to note that genistein suppressed the nude mice xenografts in ER-positive and negative breast cancer cells (49). Investigations on the mechanism of action of phytoestrogens and their combination in chemo- and radiation therapy might be a fruitful approach to the design of improved breast cancer therapies.

Acknowledgements

This work was supported, in part, by research grant DAMD17-97-1-7035 from the U.S. Army Medical Research and Material Command (S.B.) and CA42439 from the National Cancer Institute (T.T.).

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APPENDIX B

Stabilization of Cellular and Recombinant Estrogen Receptors as 4S Monomeric Form in the Presence of Genistein

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ABSTRACT

Genistein exerts growth stimulatory effects on estrogen receptor (ER)-positive MCF-7 breast cancer cells, although it is believed to have beneficial effects in protecting women from breast tumors. To differentiate the mechanisms of action of genistein from that of estradiol (E_2), we used [^{14}C]-labeled genistein and measured its interaction with ER from MCF-7 cells and human recombinant ERα and ERβ. At nanomolar concentrations of [^{3}H]-E_2, E_2-bound ER was detectable in sucrose density gradients, whereas micromolar concentration of [^{14}C]-genistein was required for detecting genistein-bound ER. Recombinant ERα sedimented as a 7S dimer in the presence of [^{3}H]-E_2. In contrast, a 4S monomeric form of ERα was observed in the presence of [^{14}C]-genistein in sucrose gradients. Similar results were obtained for ERβ as well as a 1:1 mixture of ERα and ERβ. Furthermore, an 8S form of ER was observed when cytosol from MCF-7 cells was incubated with [^{3}H]-E_2. However, with [^{14}C]-genistein, only the 3.5S form of the receptor was observed. Our results indicate that genistein-bound ER is deficient in the dimerization function generally observed with E_2- bound ER.

Key words. genistein, estrogen receptor, sucrose density gradients, dimerization

INTRODUCTION

Epidemiological studies suggest that consumption of soy-based foods protects women against breast cancer (1). This beneficial effect is often attributed to the presence of the isoflavone genistein in soy beans (1). Paradoxically, genistein has also been reported to have weak estrogenic effects in the reproductive systems of animals (2) and MCF-7 breast cancer cells (3) owing to its structural similarity to the endogenous hormone, 17-β-estradiol (E_2). Estrogens act through estrogen receptors, ERα and ERβ (4). In vitro binding assays showed that genistein competed with [^{3}H]-E_2 to bind to ERα and ERβ with ~20- and 3-fold lower affinity, respectively, compared to unlabeled estradiol (5). However, transactivation potency through the consensus ERE, in the presence of genistein was 45-50% higher for ERα than ERβ, suggesting that factors other than receptor affinity may play an important role in the complex biological effects of this compound (6).

Both ERα and ERβ are capable of forming homo and/or heterodimers in the presence of E_2 (7). E_2-induced dimerization of ER is part of the mechanism of action of ER at its consensus binding sequence, estrogen response element (ERE), leading to stimulation of gene expression (8). Selective estrogen receptor modulators such as tamoxifen and ICI 164,384 are known to alter the monomer-dimer equilibrium of the ER in a manner different from that of E_2, thus evoking diverse estrogenic/antiestrogenic responses in target cells (9, 10). We therefore hypothesized that the differences in the action of genistein and E_2 on estrogenecity may in part be due to their differential effects on the dimerization of ER.

MATERIALS AND METHODS

Materials

Full length recombinant human ERα and ERβ were purchased from Panvera Corp. (Madison, WI). [^{3}H]-E_2 was from NEN Life Science Products, Inc. (Boston, MA). The specific activity of [^{3}H]-E_2 was 72 Ci/mmol. The radioligand [^{14}C]-genistein was obtained from Moravek Biochem, Inc. (Brea, CA). The specific activity of the compound was 16 mCi/mmol. [^{14}C]-genistein was diluted in 10% ethanol before use in our experiments. ERα and ERβ were diluted from the stock in a buffer containing 10 mM Tris, 1 mM EDTA, 2 mM dithiothreitol, 10% glycerol, and 1 mg/ml BSA. The concentration of ERα and ERβ used in this study was 4213 and 2421 pmoles/ml, respectively, as reported by the manufacturers, with a protein concentration of 0.480 and 0.540 mg/ml. A 1000-fold dilution of this receptor stock provided a working solution with ER levels comparable to uterine tissues or MCF-7 cells (11, 12). Dextran coated charcoal (DCC) assays (12, 13) showed about 10-fold lower amount of the receptor from that reported by the manufacturer.

Sucrose density gradient analysis of human recombinant ERα and ERβ; and MCF-7 cellular ER

Recombinant ERα or ERβ was incubated with 10 μM [^{14}C]-genistein for 3 h at 4°C. After incubation, unbound genistein was removed by incubating with DCC suspension (0.05%) for 10 min at 4°C. The samples were then centrifuged and the supernatant loaded on a 10-30% linear sucrose gradient in TEDG buffer containing 10 mM Tris, 1 mM EDTA (pH 7.5), 1 mM dithiothreitol (DTT) and 10% glycerol. Gradients were centrifuged in a Beckman SW60 rotor (Beckman Instruments, Inc. Palo Alto, CA) at 250,000 X g for 16 h. Fractions were collected into 5 ml scintillation fluid and the bound radioactivity was quantified using a scintillation counter. Nonspecific binding was determined in parallel samples incubated with a 50-fold molar excess of unlabeled genistein. The unlabeled genistein had to be limited to 50-fold excess because of low solubility of genistein. In separate experiments, ER was incubated with 10 nM [^{3}H]-E_2 alone or in combination with a 200-fold molar excess of unlabeled E_2 and processed in a similar fashion as described for genistein.

MCF-7 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) with 100 μg/ml penicillin, 100 μg/ml streptomycin, 2 μg/ml insulin, and 10% fetal bovine serum (FBS). For two weeks before each experiment, cells were grown in phenol red-free DMEM. FBS was treated with DCC to remove endogenous estrogens. To prepare cellular ER, MCF-7 cells (~200 x 10^6) were harvested into PBS, sonicated in TEDG buffer and centrifuged at 100,000 X g in a Beckman SW60 rotor for 45 min. The cell lysate (250 μl) was incubated for 3 h at 4°C with 10 μM [^{14}C]-genistein or 10 nM [^{3}H]-E_2. The samples were then processed in a similar fashion as described for recombinant ER. Results are representative of 2-3 experiments.

RESULTS

Sucrose density gradient technique is a convenient approach to study multimeric forms of estrogen receptors under various experimental conditions. In low salt sucrose density gradients, uterine ER has been shown as a 7-9S form or as a monomeric 4S form (13). To understand the differences in the structural and/or conformational state of recombinant ER bound to estradiol and genistein, we conducted sucrose density gradient analysis in the presence of these two ligands. [^{3}H]-E_2 and [^{14}C]-genistein were used to monitor the sedimentation profile of ER. A series of gradients were conducted at 1-10 nM [^{3}H]-E_2 and 1-10 μM [^{14}C]--genistein. Recombinant ER sedimented as a distinct peak with 1 to 10 nanomolar concentrations of [^{3}H]-E_2, although 10 nM concentration yielded the maximal peak. In contrast, 10 μM genistein was needed to achieve a discernible ER peak. As shown in Figs. 1A and B, ERα or ERβ sedimented as the dimeric 7S form in the presence of E_2. In contrast, genistein bound to ERα or ERβ sedimented as the monomeric 4S form in sucrose gradient. We also examined the sedimentation profile of a 1:1 mixture of ERα and ERβ. In this case also ER bound to [^{3}H]-E_2 sedimented as the dimeric form, while ER bound to [^{14}C]-genistein sedimented as 4S form (results not shown).

In order to determine if the conformational changes observed between genistein- and E_2-bound recombinant ER also apply to ER present in breast cancer cells, we used ER from MCF-7 cells in our sucrose gradient analysis. As shown in Fig. 1C, cellular ER sedimented as an 8S dimer with [^{3}H]-E_2. In contrast, genistein bound ER sedimented as a monomeric form, smaller than the recombinant ER monomer. It is possible that genistein-bound 4S ER is more susceptible to degradation due to the proteases present in the cellular extract.

DISCUSSION

We hypothesized that the weak estrogenicity of genistein may have contribution from its inability to form homodimer of ER or heterodimer of ERα and ERβ. Our results demonstrate that recombinant and cellular ER bound to [^{3}H]-E_2 sediments as a dimer in sucrose density gradients, while ER bound to genistein sediments as a monomer. This deficiency in dimerization may be important in the mechanism of action of genistein.

Early studies characterized ER as a multiprotein complex containing the heat shock proteins hsp90 and hsp70, with a sedimentation constant of 7-9S in sucrose density gradients (14). Even though low salt sucrose gradient centrifugation shows the high molecular forms of ER in the presence of E_2, ligand binding is believed to induce dissociation of heat shock proteins and form an ER dimer that binds to ERE to activate gene expression (14).

Although it is widely accepted that ER binds to the consensus ERE as a dimer and activates transcription, there are reports of ER monomer binding to ERE (15, 16). For example, a recent study shows that the DNA-binding domain of ER can bind to the pS2 ERE half sites as a monomer, although with lower affinity than a dimer (15). Therefore, it is possible that genistein-bound ER monomer can activate transcription through certain ERE sequences such as the pS2 ERE. In fact, several investigators have used pS2 expression as a marker of estrogenecity and showed an increase in pS2 mRNA levels after genistein treatment (17).

Transcriptional regulation by genistein-bound ER may also be mediated by other factors involved in receptor activation. These include: DNA-dependent dimerization of ER at the ERE site (15), binding of ER with other transcriptional factors or accessory proteins (18), and interaction of ER with alternative estrogen signaling elements (19). Indeed, the agonistic responses of the antiestrogen tamoxifen are known to be mediated by heterodimer of ER and AP1 transcription factors fos and jun, binding to the AP1 response element (19). Furthermore, genistein has been reported to facilitate ER binding to coactivators and increase transactivation of a reporter gene (20). However, the accessory proteins in the MCF-7 cellular extract may be limited or inefficient to induce dimerization of ER in the presence of genistein.

In summary, genistein appears to be a weak estrogen at physiologically achievable concentrations (21). Our results show that a possible factor for lower estrogenic potential of genistein compared to estradiol might be their differential effects on ER dimerization.

Acknowledgements

The authors gratefully acknowledge the financial support provided by the U.S. Army Medical Research and Material Command to Srivani Balabhadrapathruni, (Grant No. DMD17-97-1-7035) and by National Cancer Institute to Thresia Thomas (Grant No. CA42439).

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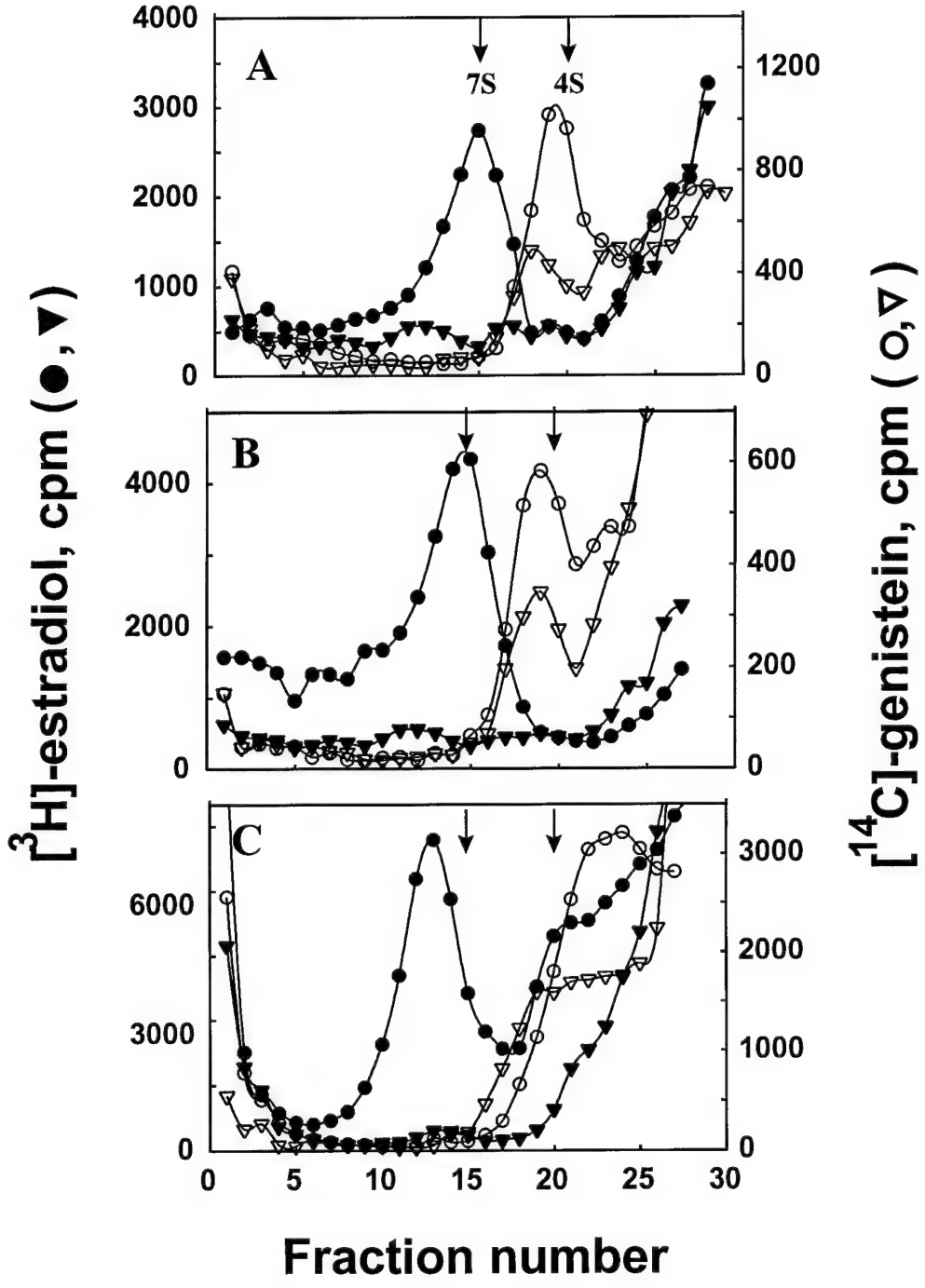
Figure Legend

Fig. 1. Sucrose density gradient profile of (A) recombinant ERα bound to [^{3}H]-E_2 or [^{14}C]-genistein, (B) recombinant ERβ bound to [^{3}H]-E_2 or [^{14}C]-genistein and (C) MCF-7 cellular ER bound to [^{3}H]-E_2 or [^{14}C]-genistein. ER was incubated with [^{3}H]-E_2 or [^{14}C]-genistein for 3 h and treated with dextran coated charcoal to remove free ligand. ER was analyzed in 10-30% linear sucrose gradients. [^{14}C]-labeled proteins γ-globulin (7S) and ovalbumin (4S) were used as markers of sedimentation constants. Binding in the presence of 10 nM [^{3}H]-E_2 (•) or 10 μM [^{14}C]-genistein (o) and non-specific binding (∇, ▼) in the presence of 200- or 50-fold excess of the respective unlabeled compound are shown.


A
7S
4S
B
C
4000
3000
2000
1000
0
1200
800
400
0
600
200
6000
3000
0
5
10
15
20
25
30
Fraction number
[3H]-estradiol, cpm (●,▼)
[14C]-genistein, cpm (○,▽)

APPENDIX C

EFFECTS OF GENISTEIN AND STRUCTURALLY RELATED PHYTOESTROGENS ON CELL CYCLE KINETICS AND APOPTOSIS IN MDA-MB-468 HUMAN BREAST CANCER CELLS

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We have studied the effects of phytoestrogens (genistein, quercetin, daidzein, biochanin A and kaempferol) on proliferation, cell cycle kinetics, and apoptosis of MDA-MB-468 breast cancer cells. Genistein and quercetin inhibited cell growth with IC_{50} values of 8.8 and 18.1 μM, respectively, while the other phytoestrogens were less effective. Flow cytometric analysis showed G_2/M cell cycle arrest with 25 μM and higher concentrations of genistein. At 100 μM, genistein, quercetin and kaempferol caused accumulation of 70, 60 and 35% of cells, respectively, in G_2/M phase by 24 h. In contrast, biochanin A and daidzein were ineffective. APO-BRDU™ analysis revealed 19.5% apoptosis with 10 μM genistein, reaching 86% at 100 μM. Apoptosis by genistein was confirmed by Hoechst 33342 staining and fluorescence microscopy. With 100 μM quercetin, 47% of the cells were apoptotic, while the other bioflavonoids had little effect. Genistein treatment resulted in a biphasic response on cyclin B1: 70% increase in cyclin B1 level at 25 μM, and 50 and 70% decrease at 50 and 100 μM, respectively. In contrast, the action of quercetin involved an increase in cyclin B1 level up to 100 μM. Genistein had no effect on cdc2 level up to 50 μM concentration; however, there was a decrease in the phosphorylated form of the protein at 100 μM. Quercetin had no effect on cdc2 levels. Our results suggest that the action of genistein and quercetin involves G_2/M arrest and apoptosis in MDA-MB-468 cells. Biochanin A and daidzein, although structurally related to genistein, did not share this mechanism. Thus, structurally related phytoestrogens have discrete target sites and mechanisms in their growth inhibitory action on breast cancer cells.

The U.S. Army Medical Research and Materiel Command under DAMD17-97-1-7035 supported this work.

Our results show that genistein and quercetin are highly efficient in inhibiting the growth of MDA-MB-468 cells. Accumulation of cells in the G_2/M phase and apoptotic cell death were prominent features of the mechanism of action of both compounds. However, with genistein, inhibition of DNA synthesis does not appear to be dependent of G_2/M arrest because 10 μM genistein was able to suppress DNA synthesis by 53% whereas G_2/M arrest was not apparent at this concentration. Genistein and quercetin treatments resulted in significant accumulation of cells in the G_2/M phase of the cell cycle amounting to 70 ± 4, and 60 ± 5% ($p<0.05$, $n=6$) compared to control with 100 μM treatment for 24 h. While kaempferol and biochanin A showed growth inhibition with IC_{50} value in a similar range; kaempferol had more significant effects on cell cycle arrest than biochanin A. The order of potency of genistein > quercetin > kaempferol remains the same with antiproliferative effects as with the efficacy of cell cycle arrest.

To study the effects of phytoestrogens on apoptotic cells death, MDA-MB-468 breast cancer cells were treated with the compounds for 24 h and percentage apoptotic cells were assessed using the APO-BRDU kit (Pharmingen, CA). Treatment of cells with 10, 25, 50 and 100 μM genistein resulted in significant increase in apoptotic cells (Table 1). Also, quercetin exposure at 100 μM resulted in 47% apoptotic cells. Interestingly, other structurally related phytoestrogens, such as biochanin A and kaempferol only had minor effects on apoptosis, even though these compounds had growth inhibitory effects with IC_{50} of ~45 μM.

Phytoestrogen	**% apoptosis by 24 h treatment with:**				
	0 μM	**10 μM**	**25 μM**	**50 μM**	**100 μM**
Genistein	1.6 ± 0.6	19.5 ± 9.8*	34.7 ± 8.3*	64.3 ± 15.0*	86.0 ± 4.0*
Quercetin	1.6 ± 0.6	3.3 ± 0.4	12.3 ± 1.5*	18.1 ± 5.6*	47.2 ± 1.5*
Biochanin A	1.5 ± 0.6	1.5 ± 0.1	3.8 ± 1.3	6.1 ± 0.3*	11.2 ± 1.2*
Daidzein	1.5 ± 0.8	1.3 ± 0.1	2.4 ± 0.1	2.9 ± 0.1	1.7 ± 0.6
Kaempferol	1.9 ± 0.1	2.5 ± 1.7	3.4 ± 0.7	3.7 ± 0.7	4.8 ± 0.4*

Table I. Effect of phytoestrogens on percentage apoptosis of MDA-MB-468 cells
* Significantly different from control, $p<0.01$, $n=4$.

To further understand the genistein activated pathways that induce cell cycle arrest and apoptosis, the expression of cyclin B1 and cdc2 proteins that function during G_2/M phase transition was examined by Western blot analysis. There was a biphasic response with a 70% increase in cyclin B1 at 25 μM genistein, compared to the control. In contrast, at 50 and 100 μM genistein, cyclin B1 level was reduced to 50 and 30% of the control, respectively. Quercetin treatment showed an increase in cyclin B1, with 100 μM treatment resulting in 30% increase. Western blot analysis of cdc2 showed no change at 10, 25, and 50 μM genistein treatment. However, at 100 μM genistein, the upper band corresponding to the phosphorylated form of cdc2 showed a marked decrease. In contrast, β-actin levels were not altered. The level of cdc2 was unchanged after quercetin treatment. Thus, growth inhibition and apoptosis by structurally related phytoestrogens involves different molecular pathways.

#627 Synthetic glycoamine analogs synergize with taxol and cisplatin in inducing programmed cell death in ovarian cancer cells. Frankel, A., Glinsky, G.V., Mossine, V.V., Buckman, R., and Kerbel, R.S. *Sunnybrook Health Science Centre, University of Toronto, Toronto, Ontario, Canada, M4N 3M5, Metastat Inc., Cancer Research Center, University of Missouri, Columbia, Missouri 65201*

Resistance to chemotherapeutic agents remains one of the most significant problems in treating ovarian cancer patients. Results with a panel of ovarian cancer cell lines exposed to taxol suggest a possible mechanism of acquired drug resistance in tumors based on the response of a cell population (multicellular resistance) as opposed to classic unicellular resistance mechanisms. Upon exposure to taxol for a period of 48 hours, a panel of ovarian cancer cell lines demonstrated a drug- resistant phenotype when grown as three-dimensional cultures (multicellular tumor spheroids), but did not exhibit this phenotype when grown in monolayer cultures. After treatment with taxol, the cells grown as three-dimensional cultures had a greater survival, an increased proliferative potential as well as colony forming ability compared to the taxol-exposed cells grown in monolayer culture. The monolayer cultures exposed to taxol had a greatly altered cell cycle profile that was not apparent in the spheroid model. Taxol-induced apoptosis in the monolayer culture as measured by flow cytometry for concomitant detection of apoptosis and cell cycle analysis was not observed in the spheroids. The levels of the anti-apoptotic protein, bcl-x_L, decreased significantly upon taxol treatment in the monolayer culture, whereas the bcl-x_L levels in the spheroids treated with taxol remained elevated, closely resembling the levels of the control spheroids. We have studied the ability of synthetic glycoamine analogs, anti-adhesive and anti-metastatic agents, to cause a reversal of multicellular resistance to taxol-induced apoptosis or to synergize with cisplatin in inducing apoptosis in ovarian cancer cells. These glycoamine analogs act by competing for specific carbohydrate-lectin interactions, particularly those involving β-galactoside-specific lectins expressed on tumor cells. Three synthetic glycoamines (Fru-Gly, Fru-D-Leu, and Lac-L-Leu) induced programmed cell death in the ovarian cancer cell line A2780. These compounds synergized in vitro with taxol as well as cisplatin to increase the apoptotic index of ovarian cancer cells. Our results support the idea that anti-adhesives and anti-metastatic agents, synthetic glycoamine analogs, may have a translational potential in combination with chemotherapeutic drugs to treat ovarian cancer patients.

#628 Taxol induces apoptosis by a p53/p21 independent mechanism in cisplatin resistant ovarian carcinoma cells. Menendez, A.T., Laidlaw, J., Raventos-Suarez, C., Gelbert, L., Granas, A., Li, M-X., and Kramer, R. *Bristol-Myers-Squibb, Pharmaceutical Research Institute, Oncology Drug Discovery, Princeton, NJ 08543-4000*

Cisplatin and taxol are widely used in the treatment of cancer. While loss of p53 function has been correlated with resistance to DNA damaging agents, such as cisplatin, its role in the sensitivity of human tumors to taxol (an inducer of p53 and p21) has not been clearly defined. In this report we describe studies using cisplatin resistant A2780DDP human ovarian carcinoma cells that were selected by intermittent cisplatin treatment. We demonstrate that A2780DDP cells have a G to T transverse mutation in exon 5 of the p53 gene (V to F) and parental A2780S have wild-type p53. Whereas A2780DDP cells were resistant to cisplatin as compared to parental A2780S cells, taxol was equally cytotoxic in both cell lines. Although p53 and p21 levels were induced in A2780S cells after a 24 hour exposure to either cisplatin or taxol, neither p53 nor p21 were induced in A2780DDP cells under the same conditions. Cisplatin treated A2780S cells show a slow S traverse, maintain G1 arrest and undergo apoptosis after 24 hours. Cisplatin treated A2780DDP cells also showed a slow S traverse time, and undergo apoptosis but G1 was decreased; these effects required higher drug concentrations and longer drug exposure times. Taxol treated A2780S and A2780DDP cells undergo pronounced G2/M arrest and apoptosis at equal concentrations. We show in this report that taxol sensitivity (but not concomitant cisplatin resistance) in a human ovarian carcinoma cell line is independent from a clinically relevant p53 mutation.

#629 Non-steroidal anti-inflammatory drugs (NSAID) protect human T lymphoma cells against apoptosis induced by anti-cancer drugs. Azare, J., Cohen, D., and Flescher, E. *New York University Med. Ctr., Tuxedo, NY 10987, Oncology Preclinical Res., Sandoz Res. Inst., East Hanover, NJ 07936 [D.C.]*

Anti-cancer drugs can induce apoptosis in cancer cells and their removal from the cells is mediated by the efflux pump P-glycoprotein (P-gp). We have previously shown that NSAID enhance the multidrug resistance gene (MDR1) expression and function of P-gp in transformed T lymphocytes. The purpose of the present study was to assess the ability of NSAID to protect Molt-4 cells against induction of apoptic death. Aspirin and sodium salicylate at 2 mM (levels attainable in the plasma and enhancing P-gp function *in vitro*) reduced adriamycin (5 μM)- and taxol (100 nM)-induced apoptosis. For instance, aspirin reduced taxol-induced apoptosis by 82%, $P<0.0005$. These findings can be explained by enhanced removal of anti-cancer drugs from NSAID-treated cells, and suggest a potential contra-indication for the use of NSAID during lymphoma chemotherapy. (These studies have been supported by Department of the Army Grant #DAMD17-95-1-5058 and by NIOSH Grant #OH07125.)

#630 Expression of a protective TR1 gene during cell death by tumor necrosis factor and staurosporine. Chang, N-S., Mattison, J., Cao, H., Joki, N., Zhao, Y., Grasso, M., and Lee, C. *Guthrie Research Institute, 1 Guthrie Square, Sayre, PA 18840*

Transforming growth factor-β1 (TGF-β1) is known to prevent L929 fibrosarcoma cell death by tumor necrosis factor (TNF). This protection involves a rapid activation of cellular protein tyrosine kinases which apparently interrupts the TNF killing pathway. Furthermore, a TGF-β1-induced extracellular matrix protein of 46 kD appears to provide an additional signal for activation of tyrosine and serine/threonine kinases that restrict TNF killing. By expression cloning we have isolated a novel cDNA (1.4 kb), designated TR1, which encodes a putative 12.4 kD protein. Stable expression of TR1 cDNA in L929 cells protects the cells from TNF killing. Prosite analysis shows that the 12.4-kD TR1 protein contains two conservative phosphorylation sites and a motif of TGF-β family. TGF-β1 rapidly induces TR1 gene expression (a minor 1.4 kb and a major 3.0 kb mRNA transcripts) in L929 cells within 1-hr stimulation, which correlates with the induced TNF resistance in these cells. TR1 gene is also expressed in 4–6 hr by stimulation of L929 cells with TNF or staurosporine. That is, TR1 gene is expressed when L929 cells are undergoing apoptosis. These results suggest that TR1 is a protective protein against TNF- and staurosporine-mediated cell death. Western blotting analysis using antibodies against a synthetic peptide of TR1 revealed a 40-kD protein recognized by the antibodies. The 40-kD TR1 could be rapidly induced by TGF-β1 within 1 hr and by TNF in 4–6 hr, which correlates with the extent of gene expression as determined by Northern hybridization. The 40-kD TR1 protein is most likely derived from the 3.0 kb mRNA transcript. TR1 could be a tumor suppressor since L929 cells stably expressed TR1 had a reduced proliferation rate. The antiproliferative nature of TR1 renders the cells less susceptible to TNF killing, since in most cases TNF targets proliferating cells (Supported in part by NIH CA61879 and CA64423).

#631 p53 overexpression converses p21-mediated G1 arrest into apoptosis in human lung cancer cells: Clinical implication for p53 gene therapy. Kagawa, S., Fujiwara, T., Nishizaki, M., Ogawa, N., Inoue, F., Hizuta, A., Roth, J.A., and Tanaka, N. *First Department of Surgery, Okayama University Medical School, Okayama 700, Japan, M. D. Anderson Cancer Center, Houston, TX 77030*

p21, a cyclin-dependent kinase inhibitor, may be critical for p53-mediated growth suppression. To directly examine the role of p21, we introduced human p21 gene into a p53-deficient human lung cancer cell line H1299 using a p21-expressing adenoviral vector (AdCMVp21). Infection with AdCMVp21 resulted in high levels of p21 protein expression, and significantly suppressed the growth of H1299 cells. Flowcytometric analysis of DNA contents showed G0/G1 arrest of cell cycle and no apoptosis. To examine the effect of p21-mediated G1 arrest on the induction of apoptosis by p53 gene transfer, H1299 cells were sequentially infected with AdCMVp21 and AdCMVp53. Overexpression of p53 on cells arresting at the G1 phase resulted in a rapid cell death, indicative of apoptosis. Time-course-flowcytometric analysis showed that cells in the G1 phase directly underwent apoptosis without the entry into the S phase. Thus, p53-mediated apoptosis is not affected by p21-mediated G1 arrest. These results suggested that p53 expression could overcome p21-mediated G1 arrest by inducing apoptosis and that p53 gene transfer may be an effective inducer of cell death even on the resting tumor cells.

PHARMACOLOGY/THERAPEUTICS (PRECLINICAL AND CLINICAL) 9: Polyamines, Ether Lipids, Signal Transduction Inhibitors, and Antifolates

#632 A synergistic antiproliferative effect of a polyamine analogue and a triplex forming oligonucleotide (TFO) on MCF-7 breast cancer cells. Balabhadrapathruni, S., Thomas, T., Shirahata, A., and Thomas, T.J. *UMDNJ-Robert Wood Johnson Medical School, New Brunswick, NJ 08903, Josai University, Saitama 350-02, Japan*

Targeting of oligopurine oligopyrimidine sequences in the promoter regions of specific genes by TFOs is a novel strategy to inhibit the transcription of disease-related genes. Recent investigations have revealed that synthetic analogues of the natural polyamines are excellent promoters of triplex DNA. We studied the effects of a series of spermine analogues ($EtHN(CH_2)_xNH(CH_2)_yNH(CH_2)_zNHEt$; where x, y and z are 3 or 4; for spermine x =3, y =4, and z = 3; Et =ethyl) and a 37-mer TFO (targeted to the promoter region of c-myc oncogene) as individual agents and in combination on the proliferation of MCF-7 cells by [^{3}H]-thymidine uptake. This TFO exerted a 25% inhibitory effect at the 48 h time point. Bis(ethyl)spermine and norspermine (x=3, y=3, z=3) had no significant effect on [^{3}H]-thymidine uptake, whereas bis(ethyl)homospermine (x=4, y=4, z=4) exerted a 20% inhibitory effect at 5 μM concentration. Combinations of TFO with bis(ethyl)spermine and norspermine exerted no significant effect on DNA synthesis. In contrast, a combination of TFO and bis(ethyl)homospermine inhibited [^{3}H]-thymidine uptake by 90%, suggesting a synergistic action of this combination. These data indicate that selective use of polyamine analogs is a viable strategy to develop an anti-gene therapeutic approach for breast cancer.

remove the HA and prevent it from accumulating in alveolar spaces. However, HA appears to accumulate around tumors that are present in the lung tissue. When nude mice were given tail vein injections of cancer cells (MDA 231 or HS578T human breast cancer cell lines or M109 mice lung cancer), the amount of HA in the regions of experimental lung metastases was dramatically increased. This phenomenon was also found in the spontaneous lung metastases from the nude mice received human breast cancer MCF-7 cells transfected with fibroblast growth factor 1 via subcutaneous injection and from the MMTV/ras transgenic mice. The HA staining was present both inside and surrounding the tumor nodules. Interestingly, the HA staining tended to be the strongest when the tumor nodules were small (<200 cells). When dexmathesone was i.p. injected into mice to inhibit the synthesis of HA by stromal cells, the staining of HA in lung tumor nodules was reduced around the edge, however, some residual staining in the pericellular regions of tumor cells could still be detected. These results suggest that the synthesis of HA is upregulated when tumor cells start to settle-down and grow in lung tissue and the targeting cells might be both the tumor and stromal cells. The significance of this phenomenon is further investigated.

EPIDEMIOLOGY/PREVENTION 3: Biological and Biochemical Mechanisms in Prevention, Bio markers, and Intervention

#1391 Estrogenic and anti-estrogenic actions of genistein in human breast cancer cell growth mediated through the polyamine pathway. Balabhadrapathruni, S., Thomas, T., and Thomas, T.J. *Rutgers University, New Brunswick, NJ 08903, RWJ Medical School, New Brunswick, NJ 08903*

Epidemiological and clinical studies suggest potential chemopreventive effects for the phytoestrogen genistein (GEN) against breast cancer. Proliferation of estrogen receptor positive MCF-7 breast cancer cells was determined after treatment with GEN (4', 5, 7-trihydroxyisoflavone). Thymidine incorporation assay indicated that GEN significantly increased DNA synthesis at 10 μM compared to controls. In contrast, there was a 50% reduction in DNA synthesis at 25 μM, indicating an antiestrogenic role for this drug. To elucidate the mechanism by which GEN exhibits the dose-dependent estrogenic or antiestrogenic actions, its influence on enzymes of polyamine metabolism; ornithine decarboxylase (ODC), S-adenosylmethionine decarboxylase (SAMDC) and spermidine/spermine-N'-acetyltransferase (SSAT) was studied. Polyamines are cellular cations involved in cell proliferation and differentiation and their levels are regulated by estradiol in MCF-7 cells. GEN significantly increased ODC and SAMDC activity at 10 μM concentration. At growth inhibitory concentrations of GEN, however, these enzymes were inhibited. There was also a dose-dependent increase in SSAT levels with GEN treatment. These results indicate that a possible mechanism for GEN action might involve a polyamine pathway, eliciting growth promotive and suppressive effects depending on the concentration of the drug.

#1392 Breast cancer prevention through the targeted destruction of breast epithelial cells. McKenzie, K.E., Adey-Hedican, C., Aguilar-Cordova, E., Woo, S.L.C., and Sukumar, S. *Johns Hopkins Oncology Center, Baltimore, MD 21205, Baylor College of Medicine, Houston, TX 77030, Mount Sinai Medical Center, New York, NY 10029*

We are developing a novel prophylactic treatment for breast cancer. Our approach is based on the observation that more than 95% of invasive breast cancers arise from the epithelial subset of breast cells, which line the ducts of the mammary gland and are therefore easily accessible to cytotoxic substances injected via the nipple. The targeted destruction of breast epithelial cells should render the gland resistant to tumorigenesis while leaving the macro structure of the breast intact. We are evaluating mammary epithelial cells destruction by the direct cytolysis via lytic vaccinia virus or the indirect cytolysis via the Herpes thymidine kinase carried in adenoviral vectors (Ad-HTK). In the Ad-HTK protocol, ganciclovir is incorporated into the cellular DNA to produce a cytotoxic effect following cell division. We used a carcinogen-induced rat mammary tumor model to assess the degree of protection from tumorigenesis afforded by each of these methods. The vaccinia protocol resulted in up to 75% destruction of the ductal tree within 48 hours. The Ad-HTK protocol resulted in minimal epithelial cell destruction but significant protection from tumorigenesis. This protection may occur because the proliferative requirement of the Ad-HTK protocol makes it more cytotoxic for the cells that are most likely to form tumors.

#1393 Enhancement of immune function in mice fed with high doses of soy daidzein. Zhang, R., Li, Y., and Wang, W. *Department of Biological Sciences and Biotechnology, Tsinghua University, Beijing 100084, China, Cancer Research Center of Hawaii, University of Hawaii, 1236 Lauhala Street, Honolulu, HI 96813*

High soy consumptions leading to high exposures of soy isoflavones have been associated with a reduced risk of cancer. As part of a study focusing upon the chemopreventive mechanisms, we have investigated the modulating effects of daidzein, one prominent and more bioavailable isoflavone in soy foods, on the murine immune function. Swiss mice were fed daily for 7 consecutive days with daidzein at various doses. At high doses of both 20 and 40 mg/kg, daidzein exerted a stimulatory effect on nonspecific immunity, as shown by increases in phagocytic response of peritoneal macrophage and thymus weight. An augmentation of spleen IgM-producing cells against sheep red blood cells demonstrated an activation of humoral immunity. An enhanced cell-mediated immunity (increased lymphocyte proportion of peripheral blood *in vivo* and lymphoproliferation *in vitro*) was also observed. However, no immunostimulatory effects were found when administrated with daidzein at low dose of 10 mg/kg. These results demonstrate for the first time that daidzein at high doses is able to enhance *in vivo* several immunological functions, and suggest a novel approach to understanding the mechanism(s) by which soy foods contribute to observed cancer prevention.

#1394 Selenocompounds induce a redox modulation of protein kinase C in the cell compartmentally independent from cytosolic glutathione. Gundimeda, U., Chen, Z., and Gopalakrishna, R. *University of Southern California, Los Angeles, CA 90033*

Since selenite, selenodiglutathione, and selenocystine can induce a redox modification of protein kinase C (PKC) in the test tube, we have determined whether this modification can occur in the cell where the environment is highly reducing in nature and its role in cancer chemoprevention. In JB6 cells treated with chemopreventive doses (0.5 to 2 μM) of these agents, only a slight and transient redox inactivation of PKC was observed. However, when the cells were serum starved or pretreated with 2-deoxyglucose to decrease cellular reducing equivalents, the effect of selenocompounds was more pronounced. When the cellular glutathione was depleted by pretreating cells with buthionine sulfoxamine for 24 h, there was no change in the effects of selenocompounds on PKC modification as well as on PMA induction of ornithine decarboxylase suggesting that glutathione is not required for mediating these selenium effects. This also argues against the cellular distribution of PKC predominant to the cytosol. In the crude cell extracts, PKC was inactivated by selenite (IC_{50} = 0.05 μM), which was reversed by NADPH. A NADPH-dependent protein disulfide reductase was responsible for reversing PKC modification. Conceivably, selenocompounds induce a redox modification of PKC compartmentally independent from cytosolic glutathione, which at least in part, may mediate their antitumor promoting actions.

#1395 Ellagic acid induces oxidative inactivation of protein kinase C by modifying both catalytic and regulatory domains. Chen, Z., Gundimeda, U., and Gopalakrishna, R. *University of Southern California, Los Angeles, CA 90033*

Ellagic acid is a polyphenol found in fruits and vegetables, and has been shown to have cancer chemopreventive activity. The mechanism of its antitumor promoting activity is not known. Since protein kinase C (PKC) serves as a receptor for tumor promoters as well as can be oxidatively modified by phenolic compounds, we have determined whether ellagic acid can directly regulate this enzyme. When PKC was preincubated with ellagic acid and then assayed with a low (5 μM) or high (100 μM) concentration of ATP, the enzyme was inhibited with IC_{50} of 1 and 4 μM, respectively. Detailed studies revealed that this was due to an irreversible inactivation of the enzyme caused by a redox modification. Ellagic acid-iron complex can inactivate PKC without the need for other metals. Both catalytic and regulatory domains of PKC were modified by ellagic as determined by the loss of kinase activity and phorbol ester binding. But the catalytic domain was 3-fold more sensitive. The thiol agents and vitamin C inhibited this inactivation. In JB6 cells, ellagic acid at low (<1 to 5 μM) concentrations induced a modification of PKC which was reversed by an endogenous reduction mechanism, while at higher (5 to 25 μM) concentrations, it induced an irreversible inactivation. Taken together these results suggest that the antitumor promoting action of ellagic acid may be mediated in part by inducing a redox modification of PKC.

#1396 Ascorbate in the presence of glutathione results in antioxidant activity and cell cycle arrest. Bijur, G., Briggs, B., and Williams, M. *Dept. of Medical Microbiol. and Immunol., Dept. of Pathology, The Ohio State University, Columbus, OH 43210*

Ascorbic acid (AA) is a vitamin which has both anti and pro-oxidative activities. To elucidate the mechanism(s) of AA activity during oxidative stress (OS) we used the Chinese hamster ovary fibroblast cell line, AS52, which lacks the normal X linked hypoxanthine-guanine phosphoribosyl transferase (*hprt*) gene, but contains a transfected *E. coli* guanine phosphoribosyl transferase (*gpt*) gene. OS was induced by a xanthine oxidase radical generating system (RGS). Our studies show that cells treated with RGS exhibited a decrease in cell survival (CS) versus untreated cells (70%±14s.d. and 100%±20s.d. respectively) and an increase in mutation frequency (MF) (60.2 and 19.6 mutants per 10^6 clonable cells, respectively). Pretreatment with 50 μM AA followed by RGS increased CS to 94%±12s.d. and decreased the MF to 21.8. Cotreatment with RGS and buthionine sulfoximine (BSO), an inhibitor of glutathione (GSH) synthesis, resulted in decreased CS, (56±9.5s.d.). Cotreatment with RGS, BSO and AA resulted in a dramatic decrease in CS. Flow cytometric analysis demonstrated that pretreatment with AA results in cell cycle arrest at the G2 phase. This study suggests that under OS, AA is an effective antioxidant but lacks this ability when GSH synthesis is inhibited. The protective capability of AA maybe due to its ability to cause cell cycle arrest which allows recovery from DNA damage induced by oxidative stress.

provided a significant amount of information about the presence or absence of telomerase activity, it does not indicate if all cells within a tumor have telomerase activity or if only a subset does. We used fluorescent-labeled primers and an in situ PCR to detect telomerase activity at the single cell level. In various types of tumor derived cell lines, bright fluorescent signals were observed in the nucleus. The incidence of bright fluorescence in nuclei ranged from 14% to 67%, indicating the heterogeneous expression of telomerase activity. In normal resting lymphocytes only weak fluorescence was detectable, while PHA-stimulated lymphocytes showed bright punctate nuclear fluorescence in the nucleus. These findings suggest that the in situ PCR assay is able to detect telomerase activity at the cellular level in tumor but not normal cells and will be applicable for use on cytological samples obtained from cancer patients. The in situ TRAP assay may provide added value to cytological examination in clinical cancer diagnosis.

#3387 DNA-interacting drugs that down-regulate telomerase and interact with telomeric repeats in human cancer cells. Raymond, E., Soda, H., Sharma, S., Sun, D., Izbicka, E., and Von Hoff, D.D. *Translational Research Laboratory, Cancer Therapy and Research Center, Institute for Drug Development, San Antonio, TX 78245 USA*

Telomerase and telomeres have been proposed as ideal targets for limiting the proliferative life-span of human cancer cells. We evaluated whether DNA-targeting drugs (DNA alkylating agents, intercalating drugs, and topoisomerase I and II inhibitors) modulate telomerase activity and interact with telomeric sequences in human MCF-7 breast cancer cells. Telomerase activity of viable cells was measured in CHAPS extracts at days 0, 4, 8, 14 and 21 after drug exposure, using an improved conventional telomerase assay. Standardization of the number of cells, protein concentrations, and telomerase reactions allowed the quantification of telomerase activity at concentrations ranging from IC_{20} to IC_{70}. Moreover, while cis- and trans-platinum compounds do not interact directly with telomerase in cell-free systems, they interact with $(TTAGGG)_3$ telomeric repeats and can prevent telomerase-mediated primer extensions. Other alkylating or intercalating agents decreased telomerase activity at high concentrations, whereas topoisomerase I and II inhibitors did not modulate telomerase activity. Identification of mechanisms that temporarily downregulate telomerase activity and understanding the interactions between platinum compounds and telomeric DNA in whole cell systems may allow us to determine the optimal clinical situation for testing specific telomerase inhibitors in clinical trials.

#3388 Growth inhibitory effects of a telomeric oligonucleotide on MCF-7 breast cancer cells. Balabhadrapathruni, S., Thomas, T.J., Gallo, M., and Thomas, T. *UMDNJ-Robert Wood Johnson Medical School, New Brunswick, NJ 08903*

Telomerase, a reverse transcriptase that maintains the length of telomeres, is present in cancer cells and not in adjoining normal tissues. Guanine-rich structure of telomeres raises the possibility that G-rich oligonucleotides might inhibit telomerase activity. We studied the effect of a $(TTAGGG)_n$ oligonucleotide on the growth of MCF-7 breast cancer cells by [^{3}H]-thymidine incorporation assay. Treatment of the cells with 50 and 100 nM oligonucleotide for 24 h and 48 h did not change DNA synthesis significantly. In contrast, 72 h of treatment with 100 nM oligonucleotide resulted in 78% inhibition of DNA synthesis. At 250 nM, 86% inhibition was observed. A control oligonucleotide with scrambled sequence had no inhibitory effect. Evaluation by light microscopy indicated that cells were not undergoing apoptosis after treatment with the oligonucleotide. Hence, we measured estrogen receptor (ER) protein level as an indicator of differentiation. Dextran coated charcoal assay of ER indicated a 2-fold increase in ER level after 72 h of treatment with $(TTAGGG)_n$ oligonucleotide. These results show that the oligonucleotide targeted to inhibit telomerase is an effective growth inhibitory agent against breast cancer cells. Its mechanism of action may include the induction of cell differentiation.

#3389 Antisense telomerase treatment; induction of two distinct pathways, apoptosis and differentiation. Kondo, S., Tanaka, Y., Kondo, Y., Hitomi, M., Stacey, D.W., Barnett, G.H., Ishizaka, Y., Liu, J., Haqqi, T., Nishiyama, A., Cowell, J.K., and Barna, B.P. *Cleveland Clinic Foundation, Cleveland, OH 44195, Tokyo Metropolitan Institute of Medical Science, Tokyo 113, Japan, International Medical Center of Japan, Tokyo 162, Japan*

Telomerase, the enzyme that elongates telomeric DNA $(TTAGGG)_n$, may be essential for cellular immortality and oncogenesis. To investigate the effect of inhibition of telomerase on tumor cells, we transfected the antisense vector against the human telomerase RNA into human malignant glioma cells exhibiting telomerase activity. After 30 doublings, some subpopulations of transfectants expressed a high level of interleukin-1β-converting enzyme (ICE) protein and underwent apoptosis. In contrast, other subpopulations also showed enhanced ICE protein, but escaped from apoptotic crisis and continued to grow, although their DNA synthesis and invasive activity were reduced. Surviving cells demonstrated increased expression of glial fibrillary acidic protein (GFAP) and decreased motility, consistent with a more differentiated state. These cells also contained enhanced expression of the cyclin-dependent kinase inhibitors (CDKIs), p21 and p27, in contrast to apoptotic cells. Treatment of surviving non-apoptotic cells with antisense oligonucleotides against p27, but not p21, induced apoptotic cell death, suggesting that p27 may have protected differentiating glioma cells from apoptosis. These data show that treatment with antisense telomerase inhibits telomerase activity, and subsequently induces either apoptosis or differentiation. Regulation of these two distinct pathways may be dependent upon the expression of ICE or CDKIs.

#3390 Telomere maintenance and apoptosis in metastatic cancer cells. Glinsky, G., Glinsky, V., Holt, S., and Shay, J. *Cancer Research Center, University of Missouri-Columbia, MetaStat, Inc., Columbia, MO 65201, University of Texas Southwestern Medical Center, Dallas, TX 75235*

No systematic experimental evidence has been presented describing the mechanisms of telomere maintenance and regulation of apoptosis in metastatic cancer cells. Here we investigated these two phenomena in a panel of 9 murine and human cancer cell lines exhibiting different metastatic potential. The TUNEL assay, DNA fragmentation analysis, and viability assay were used for apoptosis analysis. Telomere length was estimated from TRFs analysis of *AluI* or *HinfI* digested DNA and telomerase activity was analysed using a PCR-based TRAP assay. Dramatic loss of telomerase activity was observed in poorly metastatic cells subjected to serum deprivation-induced apoptosis. The PCR-based assay with telomere-specific primers was employed for analysis of telomeric repeat degradation products (TRDP) in low molecular weight DNA extracts. The TRDP assay revealed an asymetrical accumulation of (CCCTAA)*n* telomere fragments in proliferating *in vitro* cancer cells, suggesting nuclease-mediated G-tail formation mechanism on chromosome ends. The enhanced degradation of telomeric DNA was correlated with increased activity of nuclear Ca^{2+} -dependent endonucleases and higher levels of apoptosis. Decreased degradation of telomeric DNA, low nuclear Ca^{2+} -dependent endonuclease activity, and diminished level of apoptosis were associated with the high metastatic potential. Our data strongly suggest that telomere maintenance is significantly disrupted during apoptosis and telomeric DNA repeats may be direct targets for apoptosis-associated endonucleases. Our findings also imply that highly metastatic cancer cells exhibit increased resistance to apoptosis and higher stability of telomere maintenance mechanisms.

#3391 Telomerase activity as a predictor of relapse in Wilms' tumor. Dome, J.S., Perlman, E.J., Adey, C., Carey, L.A., Umbricht, C.B., Varon, D., and Sukumar, S. *Johns Hopkins Oncology Center, Baltimore, MD 21205*

Wilms tumor (WT) may be divided into two histologic types, favorable and anaplastic. Treatment groups based on histology have limited value in predicting outcome since 10–15% of patients with favorable histology tumors relapse. We prospectively evaluated telomerase as a marker to predict disease recurrence in WT. A panel of 35 WTs (17 favorable histology, 10 diffuse anaplastic, and 8 focal anaplastic) was obtained from our institution and the National Wilms Tumor Study Group. Frozen sections were stained with H&E to confirm tumor presence. Telomerase activity was determined by a quantitative telomeric repeat amplification protocol (TRAP). 31/33 (94%) tumors tested positive for telomerase. The mean telomerase activity was two-fold higher in tumors with diffuse anaplasia than in those with favorable histology or focal anaplasia ($p=.06$, Wilcoxon). The 2-year relapse-free survival (RFS) rate was 60% in tumors with low telomerase activity and 30% in tumors with high telomerase activity ($p=.08$, Wilcoxon). Similar differences in RFS were seen when anaplastic tumors were excluded from the analysis, suggesting that telomerase activity predicts outcome independent of histology. Though limited by small sample size, this study suggests that high telomerase activity portends relapse in WT. This enzyme may identify patients who would benefit from more intensive therapy.

#3392 Telomerase activity in normal and tumorigenic rat hepatic epithelial cells. Golubovskaya, V., Smith, G., Presnell, S., Hooth, M., McCullough, K., Byrd, L., Irvin, C., Coleman, W., and Kaufmann, W. *Department of Pathology and Laboratory Medicine, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599*

Telomerase is a ribonucleoprotein DNA polymerase that maintains telomeric DNA repeats at chromosome ends. Telomerase expression has been implicated in human carcinogenesis and cell immortalization. To study the role of telomerase in rat hepatocarcinogenesis, we characterized telomerase activity in various rat tissues and liver epithelial cells. Testis and liver were found to be telomerase positive, spleen had low or negative telomerase activity and kidney was negative. Telomerase activity was stable with animal aging (from 2 to 18 months) but was 2-fold increased during liver regeneration induced by a 2/3 partial hepatectomy. Telomerase activity was detected in isolated normal rat hepatocytes and in 2 immortal hepatocyte lines. Telomerase activity displayed variations in a clone of rat hepatic epithelial stem cells (WB-F344). WB-F344 cells expressed telomerase at low passage levels (passage 4–9) after establishment *in vitro*. However, during further passaging *in vitro* these cells lost expression of telomerase. Expression of telomerase in tumor-derived lines of WB-F344 cells suggests there may be a role for the enzyme in rat hepatocarcinogenesis. (Supported by PHS grants CA 59495 and CA 59486).

#3393 Telomere length on specific chromosomes in telomerase positive and negative cells from different human tissues. Martens, U.M., Zijlmans, J.M.J.M., Yui, J., Poon, S.S.S., Chavez, E.A., and Lansdorp, P.M. *Terry Fox Laboratory, British Columbia Cancer Agency, Dept. of Medicine, University of B.C., Vancouver, B.C., Canada*

#2644 The effect of dietary oltipraz and genistein on DNA adducts induced by the potent mammary carcinogen dibenzo[*a,l*]pyrene in female Sprague-Dawley rats. Smith, W.A., Arif, J.M. and Gupta, R.C. *University of Kentucky, Lexington, KY 40536.*

Oltipraz a synthetic dithiolethione and genistein an isoflavone and component of soy products have both been shown to exhibit potential chemopreventive properties *in vitro* and *in vivo*. In this study we have examined the effect of these two suspected anticarcinogens *in vivo* on DNA adducts induced by the environmental carcinogen, dibenzo[*a, l*]pyrene (DBP). Female S/D rats were provided dietary oltipraz (500 mg/kg diet), genistein (100 mg/kg diet) or control diet for 7 days prior to a single oral dose of DBP (2.2 mg/kg, b.w.). The animals remained on this diet for 5 days post-DBP dosing and were euthanatized at this time. Treatment with DBP resulted in the formation of 1 major and at least 6 minor DNA adducts in the mammary gland and lung while several additional adduct spots were detected in the liver. Adducts were the highest in the mammary gland with a level of 121 ± 46 adducts/10^9 nucleotides while lung and liver adducts were 2.5–3 fold lower with levels of 44 ± 13 and 48 ± 16 adducts/10^9 nucleotides, respectively. Intervention with oltipraz or genistein produced no qualitative differences in the DNA adduct pattern and had similar effects on individual adduct levels therefore, we report only on total binding. Oltipraz inhibited DBP-DNA adducts in all three tissues by 35–48% but was most effective in the liver. Genistein, however, did not inhibit DBP-DNA adducts in the mammary gland and in fact moderately enhanced adduct levels in both the lung (27%) and liver (35%). This study indicates that oltipraz may be an effective anticarcinogen during the pre-initiation stages of carcinogenesis while genistein most likely exerts its chemoprotective effects at later stages of cancer development.

#2645 Inductions of p53, p21, and MDM2 are associated with G2/M arrests in metastatic melanoma cells treated with genistein in vitro. Rauth, S., Boddie, E., Pezzuto, J., Pisha, E., Green, A. *Dept. of Surgical Oncology, University of Illinois at Chicago, IL 60612 USA.*

Genistein,a major isoflavone of soybeans, inhibits growth and induces terminal cell differentiation in many tumor cell types. Our recent studies show that genistein significantly inhibits growth and induces differentiation in metastatic melanoma cells. We also show that sensitivity of the cells to genistein's action depended on cellular p53. Based on these observations and the report that genistein treatment causes single- and double-strand breaks in DNA, we hypothesized that p53 plays a role in genistein's action. In the present study, we sought to determine whether p53, p53-mediated p21, and MDM2 induction occurs in the DNA damage response pathways leading to cell cycle arrest following genistein treatment. Metastatic melanoma cell lines UISO-MEL-2 and UISO-MEL-4, which contain p53 with no mutations in exons 5 thru 8 of the gene, were used in our study. The results showed that genistein induced p53, p21, and MDM2 in a dose- and time-dependent manner. Inductions of p53 and its downstream effector genes were associated with cell cycle arrest at G2/M checkpoint.

(Supported by American Cancer Society National Grant)

#2646 The natural tyrosine kinase inhibitor genistein inhibits growth and adhesion of sarcoma cell lines in vitro. Rauth, S., Green, A., Das Gupta, T.K., and Bornstein, A. *Dept. of Surgical Oncology, University of Illinois at Chicago, IL 60612 USA.*

Genistein (4',5,7 trihydroxyisoflavone) demonstrated strong growth inhibitory effects against several cancers both *in vitro* and *in vivo*. Our recent studies showed that it inhibited growth and induced differentiation in metastatic melanoma cell lines. We also showed that the chemosensitivity of the cells depended on the status of cellular p53. In the present study, we investigated genistein's effects on sarcoma cell lines in culture. The fibrosarcoma cell line HT1080 (p53-negative), the osteosarcoma cell line SAOS-2 (p53-negative), and the Ewing's sarcoma cell line UISO-ES3 (p53-positive) were used in our study. Those cells were treated with different concentrations of genistein for different time periods. The results showed that genistein significantly inhibited cell adhesion and growth in a dose- and time-dependent fashion. Treatment with 100 μM of genistein resulted in complete inhibition of cell adhesion and growth in 24 hrs. Exposure to 200 μM concentrations resulted in loss of adhesion of 50% of the cells at 2 hrs., and, by 6 hrs. after treatment, more than 95% of the cells were detached. The molecular mechanisms by which genistein mediates its effects in sarcoma cell types remain to be elucidated.

(Supported by American Cancer Society National Grant)

#2647 Protection against DMBA-induced breast cancer in female rats by life time soy consumption. Hakkak, R., Korourian, S., Ronis, M.J., Irby, D., Kechelava, S., Shelnutt, S.R., and Badger, T.M. *Arkansas Children's Nutrition Center, Departments of Pediatrics and Pathology, University of Arkansas for Medical Sciences, and Arkansas Children's Hospital Research Institute, Little Rock, AR 72202.*

Breast cancer is one of the leading causes of cancer death among women. Epidemiologic studies have suggested a relationship between diets high in soyfoods and a low incidence of breast cancer. It has been demonstrated that neonatal injection of genistein, the major isoflavinoid phytoestrogen found in soy beans, results in chemoprotection against DMBA-induced mammary carcinogenesis in a rat model. Since most of the soy consumption is through diet, it is important to know if soy diets have similar effects as injections of purified isoflavone. In the present study, female Sprague-Dawley were maintained on AIN-93 diet which sources of the protein were casein (control) or Isolate Soy Protein four weeks prior to breeding and continued throughout the study. At day 50 of age, all female pups from both diets received via gavage 80 mg/kg dimethylbenz[a]anthracene (DMBA). All rats were palpated twice weekly for mammary tumors and they were killed when 100% rats on the casein diet developed tumors. Rats on the soy diet took a longer period of time for mammary tumors to develop compared to rats on the casein diet (2 weeks). At end of the experiment rats on soy diet had an 84% incidence of tumors compared to 100% of rats on the casein diet. The number of tumors per rat on soy diets were less than (5.74 Vs 3.81) rats on casein diet. These results suggest that dietary soy consumption can protect against the development of DMBA-induced mammary tumors. Supported by USDA A256251-5100-001-025.

#2648 Genistein treatment causes G2/M cell cycle arrest and cyclin B1 accumulation in MCF-7 breast cancer cells: A possible mechanism for the preventive action of genistein. Balabhadrapathruni, S., Thomas, T., Yurkow, E., and Thomas, T.J. *UMDNJ-Robert Wood Johnson Medical School, and Rutgers Univ., New Brunswick, NJ 08903.*

Genistein (4',5,7-trihydroxyisoflavone) is a constituent of soy foods, the consumption of which is believed to contribute to the low incidence of breast cancer in oriental women. In order to understand the mechanism of action of genistein, we studied the effects of genistein on long-term cell growth, cell cycle phase kinetics, and cyclin B1 accumulation using MCF-7 breast cancer cells. Genistein inhibited cell growth in a dose-dependent manner, with an IC_{50} value of 50 μM as determined by total DNA content over a period of 6 days. At 100 μM concentration, genistein inhibited cell proliferation by ~90%. Flow cytometric analysis of cells treated with 100 μM genistein for 24 h showed a 3-fold increase in the percentage of cells in G_2/M phase (47%) of cell cycle. Western immunoblot analysis showed a 2- to 3-fold increase in the level of cyclin B1 protein in cells treated with genistein compared to controls at the 16 h time point. These data suggest a possible mechanism for the action of genistein involving mitotic arrest due to an accumulation of cyclin B1 in breast cancer cells. Future studies will be directed to understand the nature of genistein interaction(s) with cyclins and cyclin/CDK complexes.

This work was supported by a pre-doctoral Fellowship from the U.S. Army's Breast Cancer Research Program (DAMD17–97-1–7035).

#2649 Role of BTG2 and possibly p53 in growth inhibition by genistein in LNCaP, PC3, and DU145 human prostate cancer cells. Davies, J.A., Walden, P., and Bosland, M.C. *Departments of Environmental Medicine and Urology, NYU Medical Center, New York, NY 10016.*

The low prostate cancer rates in Asian countries may be attributable to high dietary intakes of soy. The major soy isoflavone genistein inhibits growth of human prostate cancer cell lines LNCaP, PC3, and DU145. We examined the mechanism of these growth inhibiting effects of genistein at non-cytotoxic (dye-exclusion) concentrations of 1.85–93 μM; genistein serum levels in Japanese consuming traditional diets are approx. 5 μM. Tritiated thymidine uptake was reduced dose-relatedly at all concentrations in all 3 lines. Genistein induced apoptosis in LNCaP cells (TUNEL assay). This was confirmed by flow cytometry, with 35% of cells being hypodiploid after 72 h exposure to a high dose of genistein. In contrast, genistein caused a partial G2/M block in PC3 and DU145 cells. The PC3 and DU145 lines have inactivating p53 mutations, but LNCaP cells express wild-type p53 which, in response to cellular stresses, transcriptionally activates genes promoting G1 arrest and apoptosis. Genistein, similar to etoposide as positive control, increased expression (Northern blot) in LNCaP cells of the antiproliferative p53 target gene BTG2 in a dose-related fashion with a maximum after 2 h exposure. PC3 and DU145 cells did not express BFTG2. These data suggest involvement of p53 in growth inhibition of LNCaP but not PC3 or DU145 cells by genistein. (Supported by CA72290 and CaP CURE.)

#2650 Potential chemopreventive properties of glutathione conjugate of benzylselenocyanate against colon carcinogenesis in rats. Reddy, B.S., Kawamori, T., Rosa, J., and El-Bayoumy, K. *American Health Foundation, Valhalla, NY 10595.*

The major goal of this study was to develop organoselenium compounds with maximal chemopreventive efficacy yet lowest possible toxicity. The chemopreventive efficacy of selenium compounds depend on the form in which they are administered, suggesting that their metabolism is important in exerting their biological effects. Our preliminary results indicate that glutathione conjugates of chemopreventive organoselenium compounds, benzylselenocyanate (BSC) and 1,4-phenylenebis (methylene)selenocyanate (*p*-XSC) are putative metabolites that may be responsible for their biological effects. The present study was designed to evaluate the potential chemopreventive activity of glutathione conjugate of BSC (BSe-SG) against colonic aberrant crypt foci (ACF) which are premalignant lesions. BSe-SG and BSC were synthesized in our laboratory. Male F344 rats were fed the control and experimental diets containing 10 ppm BSC (4.1 ppm Se) and 10 and 20 ppm BSe-SG (1.8 and 3.6 ppm Se) starting one week prior to the first of two weekly s.c. injections of azoxymethane (15 mg/kg body wt.) and until the animals were killed 8 weeks later for ACF evaluation. Administration of BSC and BSe-SG significantly suppressed the colonic ACF. Additionally, the degree of inhibition of ACF is more pronounced with BSe-SG in a dose-dependent manner

3D structure, but resistant to the peptidases (half life of about four days in complete medium at 37 °C). In three out of three cellular systems tested so far, this new peptide is approximately 10 times more potent than the original peptide. Biochemical studies *in vitro* to more quantitatively assess the binding specificity of our peptides with their target proteins are under way.

#124 IL-2-receptor dependent cytotoxicity of recombinant human RNasel-IL-2 fused protein for human cancer cells. Ueda M., Psarras K., Kitajima M., Seno M. and Komatsu S. *Dept. of Surgery Keio Univ. Tokyo 160, Faculty of Engineer. Okayama Univ. Okayama 700 and Dept. of Mol. Biol. National Institute of Agr. Resources, Tsukuba 305, Japan.*

To develop a new Missel' therapy targeted at growth factor receptor and composed of just human proteins, we have fused the gene of human pancreatic RNasel (hpRNase) to that of human IL-2 and studied the cytotoxic effect for leukemic cells, which are associated with hyperproduction of IL-2 receptors (IL-2R). A hybrid human protein was engineered by fusing the genes encoding hpRNasel and hIL-2, was isolated and refolded from E. coli inclusion bodies, and was purified to homogeneity. The hpRNase-IL-2 inhibited protein synthesis in MJ, a malignant T lymphocyte, hyper-producing high affinity IL-2R with an IL_{50} of 2×10^{-8} and in OKM-2T, which expresses about 2-fold fewer IL-2Rs compared to MJ, with an IL_{50} of 2×10^{-7}, whereas no inhibition was detectable for receptor deficient control cells or hpRNasel alone. A molar excessed IL-2 and anti-IL-2R antibody blocked the inhibition of protein synthesis dose dependently. Recombinant hpRNasel-growth factor fused protein may be useful for human cancer cells overexpressed growth factor receptors.

#125 Inhibition of HIV-1 integrase by acyclic nucleoside phosphonates. Tramontano, E., Loi, A.G., Franchetti, P., Grifantini, M. and La Colla, P. *Dipartimento di Biologia Sperimentale, Università di Cagliari, Italy; Dipartimento di Scienze Chimiche, Università di Camerino, Italy.*

Integrase (IN) is an essential enzyme in the HIV-1 cycle and, therefore, is an attractive target for antiviral strategies. Recently, it has been shown that: i) IN can bind nucleosides and nucleotides analogs; ii) nucleotides but not nucleosides are able to inhibit IN activities at 50–250 μM; iii) the phosphorylation state does not affect the inhibitory activity; iv) modifications on the sugar moiety convert inactive nucleotides into IN inhibitors. These observations suggested the existence of a specific nucleotide binding site on the HIV-1 IN that interacts more selectively with the sugar moiety rather than with the base or phosphate residues. Among nucleotide analogs are acyclic nucleoside phosphonates, a class of derivatives with broad spectrum antiviral and antitumor activity. In this study we show that acyclic nucleoside phosphonates such as PMEA, PMPA, PMEG and PMPG are able to inhibit both 3'-processing and strand transfer activities of the HIV-1 IN at micromolar concentrations. Since these derivatives are "activated" to diphosphates, we tested unphosphorylated and diphosphorylated forms and found that both inhibit IN activity, the latter being 5–8 fold more potent than the former. Furthermore, kinetic studies showed that both forms of acyclic nucleoside phosphonates are non-competitive inhibitors of the HIV-1 IN 3'-processing activity. Overall, these results suggest that: i) IN can interact also with nucleotide analogues which are devoid of a sugar moiety; ii) in this case, the phosphorylation state modulates the potency of inhibition; iii) the putative nucleotide binding site is in functional relationship with the IN catalytic site.

#126 Development of oligosaccharides as anti-angiogenic agents. Jayson, G.C., Pye, D., Gallagher, J.T. *Cancer Research Campaign Department of Medical Oncology, Paterson Inst. For Cancer Research, Withington, Manchester M20 4BX, United Kingdom.*

Several angiogenic cytokines exhibit a mandatory dependence on heparan sulfate (HS) for the ability to activate their signal transducing receptors. We have identified several structural features of HS required for the biological activation of basic Fibroblast Growth Factor (bFGF) and isolated two species of HS oligosaccharide that will inhibit bFGF. One species, heparin octasaccharides, inhibits the biological activity of bFGF through a length dependent relationship that probably inhibits receptor dimerisation and the conformational change required for the activation of bFGF. A second HS species lacks glucosamine 6-O-sulfate residues that may allow HS to engage the FGF receptor. Several oligosaccharides are active at concentrations that are clinically achievable and tolerable, and result in an inhibition of bFGF-induced motility and multiplication. This approach is directed against endothelial cell growth. To assess the relevance of oligosaccharides to cancer cells we have analysed the cell surface FGF dual receptor mechanism in a model of transformation of human colon adenoma to carcinoma in vitro. This analysis revealed that transformation is accompanied by down regulation of the proteoglycan core protein, a reduction in the affinity of HS for bFGF, a reduction in expression of FGF receptor 1 and a reduced biological response to bFGF that is partially alleviated by exogenous heparin, suggesting that the cells' response to bFGF is controlled through both the proteoglycans and the signal transducing receptor. In addition the implication is that in this model, inhibition of bFGF activity by oligosaccharides is unlikely to be of clinical benefit.

#127 Induction of apoptosis in myeloid leukemic cells by ribozymes targeted against AML1/MTG8. Matsushita, H., Kizaki, M., Kobayashi, H., Muto, A., and Ikeda, Y. *Division of Hematology, Keio University, Tokyo, Japan 160-8582 and Division of Pharmacology, Tokai University, Kanagawa, Japan 259-1100.*

AML1/MTG8 chimeric fusion gene resulting from translocation (8;21) has been proved to have critical role in the leukemogenesis of acute myeloid leukemia (AML) M2. To inhibit the proliferation of leukemic cells with AML1/MTG8, we designed two hammerhead ribozymes against AML1/MTG8, Rz1 and Rz2, which cleave AML1/MTG8 fusion transcript at 3 bases upstream and downstream from the fusion site, respectively. These ribozymes cleaved AML1/MTG8 substrate specifically in a cell-free system, and reduced the expression of AML1/MTG8 mRNA in Kasumi-1 cells carrying t(8;21) which were transfected with liposome. The proliferation of Kasumi-1 cells, but not other myeloid cell lines, was specifically inhibited by Rz1 and Rz2 with lipofection. We observed the morphological change including chromatin condensation, fragmentation and the formation of apoptotic bodies in part of Kasumi-1 cells incubated with ribozymes for 7 days. The percentage of apoptotic cells began to increased after day 3 and became maximum after day 5 (35% with Rz1 and 32% with Rz2). Apoptosis was confirmed by DNA electrophoresis which showed a pattern of DNA fragment. Flow cytometric analysis showed no increased expression of CD11b or CD14 in Kasumi-1 cells treated with ribozymes. These data suggest that Rz1 and Rz2 inhibited the growth of myeloid leukemic cells with t(8;21) by induction of apoptosis, but not differentiation, and that these ribozymes may have therapeutic potential for patients with AML carrying t(8;21).

#128 Facilitation of triplex DNA formation by bis(ethyl) (BE) polyamine analogs and breast cancer cell growth inhibition by polyamine analog-complexed triplex forming oligonucleotide (TFO). Thomas, T.J., Thomas, R.M., Antony, T., Balabhadrapathruni, S., Saminathan, M., Shirahata, A., and Thomas, T. *UMDNJ-RWJ Med. Sch., New Brunswick, NJ, Josai University, Japan.*

Targeting the promoter regions of disease-related genes by TFOs is an important strategy to inhibit their transcription. However, stability of triplex DNA under physiological pH and ionic conditions is a major impediment in advancing this approach to the clinical arena. We examined the efficacy of 2 therapeutic polyamine analogs [BE-norspermine (BE-3-3-3) and BE-homospermine (BE-4-4-4)] on the stability of triplex DNA formed from a 37-mer oligonucleotide targeted to the promoter region of c-myc gene and its target duplex. The analogs stabilized triplex DNA as detected by electrophoretic mobility shift assay. Treatment of MCF-7 breast cancer cells by analog-complexed TFO showed a synergistic growth inhibitory effect, when compared to the efficacy of polyamine analogs and TFO as individual agents. HPLC analysis showed a facile transport of the analogs into the cells. Thus, BE-polyamine analogs might be useful to enhance triplex DNA-mediated growth inhibition of cancer cells and to develop antigene therapeutics.

#129 Structural specificity effects of polyamines on the stability of RNA.DNA hybrids formed from phosphodiester (PO) and phosphorothioate (PS) oligodeoxynucleotides (ODNs). Antony, T., Thomas, T., Shirahata, A., and Thomas, T.J. *UMDNJ-RWJ Medical School, New Brunswick, NJ 08903, Josai University, Saitama, Japan.*

The success of antisense based therapeutics depends on the formation of a stable non-translational RNA.DNA hybrid. We studied the stability of two hybrids formed from a 21-mer RNA and complementary PO/PS ODNs in the presence of putrescine, spermidine and spermine. Melting temperature (Tm) of RNA.PSODN (35°C) was lower than that of RNA.-POODN hybrid (41°C). The polyamines increased Tm of the hybrids in a concentration-dependent manner, with spermine exerting the maximal stabilizing effect (ΔTm = 36°C at 100 μM spermine). Analogs of spermine ($H_2N(CH_2)_3NH(CH)_nNH_2(CH)_3NH_2$, n = 2-9; n = 4 for spermine) showed a remarkable structural specificity effect in stabilizing the hybrids. A similar pattern in the stabilizing efficacy was found with spermidine analogs. However, polyamine-mediated stabilization was more effective with POODN at 150 mM NaCl than with PSODN. The relative binding affinity of analogs paralleled their efficacy to stabilize RNA.DNA hybrids. These results provide new insights into the action of antisense ODNs which may be useful in cancer therapeutics.

#130 Inhibition of *c-myc* gene expression and leukemia cell growth by phosphorothioate triplex-forming oligonucleotides (TFOs). McGuffie, E.M., Carbone, G.M.R., Pacheco, D., Catapano, C.V. *Medical University of South Carolina, Charleston, SC 29425.*

Oligonucleotides designed to form triple helical DNA at gene promoter sites offer a means to reduce transcription and expression of proteins which contribute to uncontrolled cell growth. The *c-myc* oncogene is an attractive target for triplex-mediated inhibition since its overexpression is implicated in proliferation of leukemias, lymphomas and various other common cancers. We directed a purine-rich thioated TFO to a site immediately upstream of the P2 promoter in the human *c-myc* gene. The TFO had growth-inhibitory activity in T-cell leukemia CEM cells (90% inhibition at 10 μM), while a mismatched oligonucleotide with identical nucleotide content had minimal effect. Inhibition of proliferation was associated with reduced p64 c-myc protein expression and with the appearance of a novel 48kDa band, cross reactive with c-myc monoclonal antibody 9E10. Activity of the TFO was also associated with accumulation of cells in S-phase of the cell cycle, supporting the idea that c-myc has a role in S-phase progession. The *c-myc*-

in less than 50 μM nicotinamide, following DNA damage, ADP-ribose polymer metabolism was abrogated, NAD was consumed and never resumed normal content, and elevations in p53 levels observed in controls as measured by Western blot analyses did not occur. Furthermore, cells with low NAD content showed greatly decreased entry into apoptosis and an increased occurrence of necrosis. These results show that human mammary epithelial cells with suboptimal NAD content have altered DNA damage response pathways and suggest that optimization of niacin status may be preventive in mammary carcinogenesis. (Supported in part by NIH Grant CA65579.)

#4298 Interaction of genistein and other phytoestrogens with estrogen receptor (ER). Balabhadrapathruni, S., Thomas, T.J., Ghosh, S., Gallo, M.A., and Thomas, T. *UMDNJ-Robert Wood Johnson Medical School, New Brunswick, NJ 08903.*

Genistein, the phytoestrogen abundant in soy products, exerts estrogenic effects on ER-positive breast cancer cells. Genistein also has preventive effects on the incidence of human breast cancer. In order to understand these paradoxical effects, we analyzed the binding of estradiol, genistein, and other phytoestrogens to MCF-7 cell ER and to recombinant human ERα. The ability of phytoestrogens to displace [^{3}H]estradiol from ER was determined by competition assays. IC_{50} for displacing [^{3}H]estradiol from recombinant ERα by genistein was 60 nM compared to 900 nM for ER from MCF-7 cells. Other phytoestrogens also showed 10- to 50- fold differences in their ability to displace [^{3}H]estradiol from recombinant ER and cellular ER. Sucrose density gradient analysis of ERα bound to [^{14}C]-genistein showed a monomeric 4S form compared to a dimeric 6S form when bound to [^{3}H]estradiol. These results suggest that ER-associated proteins in MCF-7 cellular ER modulate the affinity of ER and phytoestrogens. ER bound to genistein appears to be deficient in dimerization and therefore coactivators of ER may play a dominant role in genistein-induced changes in cell growth.

#4299 Mechanism of melatonin oncostatic activity in MCF-7 breast tumor cells. Scott, A., Cosma, G., Frank, A., and Wells, R. *Dept. Environmental Health, Dept. Pathology, and Dept. Radiological Health Sciences, Colorado State University, Fort Collins, CO.*

Clinical and laboratory studies have provided evidence of oncostatic activity by the pineal neurohormone, melatonin. However, these studies have not elucidated its mechanism of action. The following series of MCF-7 breast tumor cell studies, conducted in the absence of exogenous steroid hormones, provide evidence for a novel mechanism of oncostatic activity by this endogenous hormone. We observed a 40–60% loss of MCF-7 cells after 20 hr treatment with 100 nM melatonin, which confirmed and extended previous reports of its oncostatic potency. Interestingly, there were no observed changes in tritiated thymidine uptake, suggesting a lack of effect on cell cycle/nascent DNA synthesis. Further evidence of a cytocidal effect came from morphologic observations of degenerative changes in mitochondria, accompanied by acute necrosis and autophagocytosis. Studies of mitochondrial function via standard polarography revealed a significant increase in oxygen consumption in melatonin treated MCF-7 cells. Enzyme-substrate studies of electron transport chain activities in detergent permeabilized cells demonstrated a 53% increase ($p<0.01$) in cytochrome c oxidase activity (complex IV). Finally, there was a 64% decrease ($p<0.05$) in cellular ATP levels in melatonin treated cells, as measured by chemiluminescence. These studies demonstrate an uncoupling of oxidative phosphorylation in melatonin treated tumor cells, whereby this agent exerts its cytotoxicity, and which may represent a novel mechanism of tumor prevention.

#4300 Dual roles for cyclin D1 in human breast premalignancy. Zhou, Q., Fukushima, P., De Graff, W., Miller, F.R., Mitchell, J., Stetler-Stevenson, M., and Steeg, P.S. *WCS, Lab. of Path., National Cancer Institute, National Institutes of Health, Bethesda, MD 20892; Karmanos Cancer Institute, Detroit, MI 48201.*

Cyclin D1 is overexpressed in many human in situ breast carcinoma (DCIS). To test the role of cyclin D1 in early neoplastic progression, we transfected it into human premalignant breast cell line, MCF-10A. The overexpression of cyclin D1 resulted in increased colonization in vitro, suggesting that it may functionally contribute to neoplastic progression. Interestingly, the cyclin D1 transfectants were more sensitive to γ-radiation inhibition of colonization, which was accompanied by increased apoptosis. Apo-2L, but not TNF-α, also preferentially inhibited the colonization of cyclin D1 transfected MCF-10A cells. Our results suggest that cyclin D1 overexpression may contribute to human breast neoplastic progression through promotion of colonization; However, colonization remains sensitive to induction of apoptosis by radiation or specific apoptosis signaling pathways, which may be relevant to breast cancer prevention and treatment strategies.

#4301 Diets containing whey protein or soy protein isolate protect against DMBA-induced mammary cancer in rats. Badger, T., Hakkak, R., Korourian, S., Shelnutt, S., Ronis, M., and Lensing S. *Arkansas Children's Nutrition Center, Little Rock, AR 72202.*

Proteins from milk and soybeans are reported to reduce the risk of some cancers. The present study was conducted to determine the possible preventive effects of soy protein isolate (SPI) and whey proteins on 7,12-dimethylbenz(a)anthracene (DMBA)-induced mammary cancers in rats. Adult male and female Sprague-Dawley rats were fed diets made according to the AIN-93G diet formula, except that corn oil replaced soy bean oil and the protein source was either casein, whey or soy protein isolate. Amino acids were added to equalize the essential amino acids among diets. After 3 weeks, rats fed the same diets were mated and the offspring were weaned to the same diets as the dams. At 50 days of age, female offspring (n = 19–59/group) were orally gavaged with sesame oil containing 80 mg/kg DMBA. All rats were killed when at least one palpatable mammary mass was present in 100% of the casein-fed rats. While rats fed either soy or whey had lower ($P< 0.05$) mean incidence of mammary adenocarcinoma (AC) than casein-fed rats, whey diets provided greater protection ($P<0.05$). Both soy-fed and whey-fed rats had delayed onset of mammary tumors ($P <0.05$). These results demonstrate that diets made with soy or whey protein partially prevent chemically-induced mammary AC. Diets formulated with whey protein provided significantly more protection than casein or soy-based diets. Our data further suggest that whey may be the most effective dietary protein source yet identified for mammary cancer prevention. SPI provided by Protein Technology International. Supported by USDA CRIS6251-51000-001-02S.

#4302 Inhibitory effect of dibenzoylmethane (DBM) on 7, 12-dimethylbenz[a]anthracene (DMBA)-induced mammary tumorigenesis in rodents. Lou Y-R., Lu, Y-P., Xie, J-G., Zhu, B.T., Thomas, P.E., Newmark, H.L., and Huang, M-T. *Laboratory for Cancer Research, College of Pharmacy, Rutgers University, Piscataway, NJ 08854-8020.*

DBM is a naturally occurring substance in licorice. Feeding DBM in the diet inhibited DMBA-induced mammary tumorigenesis in rodents (Carcinogenesis, 19: 1697, 1998). Oral intubation of 10 mg of DMBA to Sprague-Dawley rats produced an average of 3.4 mammary tumors per rat, and 92% of rats had mammary tumors at 17 weeks. Feeding 0.5% DBM in the diet to rats only during the initiation period inhibited the number of DMBA-induced mammary tumors by 64%, and the % tumor incidence was inhibited by 41%. Feeding 0.5% DBM in the diet during the post-initiation period inhibited the number of mammary tumors per rat by 25%, and the % tumor incidence was inhibited by 7%. There was little or no effect of dietary DBM on size per tumor when DBM was given during the initiation period. However, the average size per tumor was decreased by 36%, and tumor volume per rat was reduced by 52% when DBM diet was given during the post-initiation. Feeding DBM in the diet to mice or rats increased liver weight, decreased uterine weight, increased levels of certain hepatic cytochrome P-450 enzymes and *in vitro* DBM inhibited the binding of [^{3}H]estradiol to estrogen receptors. The above actions of DBM may play important roles for its inhibitory actions on breast tumorigenesis in rodents. (Supported by NIH grants CA49756, CA69473, and ES05022.)

#4303 Chemopreventive effects of slow release medroxyprogesterone acetate (MPA) on methylnitrosourea (MNU)-induced mammary carcinogenesis. Hill, D.L., Grubbs, C.J., Lubet, R.A., Eto, I., Kelloff, G.J., Cohen, L.A. and Steele, V.E. *University of Alabama at Birmingham, Birmingham, AL 35294, National Cancer Institute, Bethesda, MD 20892, and American Health Foundation, Valhalla, NY 10595.*

The progestin medroxyprogesterone acetate (Depo-Provera®) has demonstrated therapeutic activity in breast cancer patients who have failed tamoxifen therapy. When a slow release formulation of MPA (10 or 3 mg/Kg BW) was administered once (subcutaneous) to female Sprague-Dawley rats either 7 days prior to or 7 days following MNU, it profoundly decreased mammary carcinogenesis. In contrast, MPA (10 mg/Kg BW) given 28 days after MNU minimally decreased tumor multiplicity although it did decrease the volume of tumors that were palpable. Therefore, various doses of MPA (30, 10, 3 or 1 mg/Kg BW) were administered beginning 5 days post MNU. The two highest doses of MPA decreased tumor multiplicity > 90%, the 3 mg/Kg BW dose decreased multiplicity 66% while the lowest dose had minimal effects. The effects of this agent on hormonal levels and mammary gland morphology were also examined. The striking efficacy of MPA in rats more closely paralleled human efficacy in contrast to studies in beagle dogs and mice in which MPA reportedly caused early preneoplastic changes. Supported in part by NCI-CN-75101.

#4304 Chemoprevention of hormonal-induced carcinogenesis by antioxidants. Sharma, M. *Roswell Park Cancer Institute, Buffalo, NY 14263.*

The estrogens (17β-estradiol and estrone) and the antiestrogen tamoxifen are both known to induce DNA damage by oxidation of some of their hydroxylated metabolites to highly electrophilic quinone derivatives. As observed with other carcinogens, metabolism of estrogens and tamoxifen may occur in the liver and accumulate in susceptible tissues. If unrepaired, DNA adducts induced by such oxidation pathways have potential for endogenous tumor initiators, especially in those tissues with high peroxidase activity. Diethylstilbestrol (Des) which undergoes metabolic redox cycling to generate free radicals and form reactive quinone/semiquinone intermediate contribute to diethylstilbestrol-induced carcinogenesis. Initially, using Des-induced DNA adduct as an indicator, we tested the inhibitory effect of small molecule antioxidants, such as ascorbic acid and N-acetylcysteine on DNA damage induced by Des. Both these agents inhibit the formation of Des quinone in a dose-dependent manner. Extension of this study to inhibit the formation of quinone derivative of 3,4-estrogen catechol and 4-hydroxy tamoxifen will be described. Since antioxidant nutrients are inexpensive and high doses are remarkably nontoxic, their combined use with estrogens in postmeno-

system for the characterization of factors that influence metastasis of breast cancer cells to bone and for the testing of novel anti-metastatic agents. Parathyroid hormone related protein (PTHrP) has been implicated in bone metastasis due to its paracrine action in promoting osteoclast formation. Expression of PTHrP by tumor cells is postulated to contribute to tumor expansion in bone and hypercalcemia associated with malignancy. To examine the role of PTHrP in promoting bone metastasis in our model, we have transfected tumor cell lines with PTHrP sense and anti-sense expression vectors and quantitatively measured subsequent tumor burden in the bone using real-time PCR. Our data suggest that, while PTHrP may influence the extent of metastatic growth in bone in this model, other factors may also be important. Metastasis is a complex process in which a multitude of factors have been implicated. These factors include proteases, adhesion molecules, hormones and cytokines. We are currently determining the role of some of these factors, and using DNA microarray and differential display techniques to seek novel factors, that influence the metastasis of breast cancer to bone in our model.

#878 OVEREXPRESSION OF BONE SIALOPROTEIN IN HUMAN BREAST CANCER. Julie A Sharp, Larry W Fisher, Michael A Henderson, and Erik W Thompson, *NIDR, Bethesda, MD, Univ of Melbourne, St Vincent's Hosp, Fitzroy, Australia, and VBCRC Invasion and Metastasis Unit, St Vincent's Hosp, Fitzroy, Australia*

Bone sialoprotein (BSP), a secreted glycoprotein normally found in bone matrix and implicated in the formation of mammary microcalcifications, has been localized to human breast cancers (HBC). BSP was shown to be produced exclusively by cancer cells in a cohort of primary human breast carcinoma specimens using immunohistochemistry and in situ hybridization techniques (Sharp et al, 1999. Lab Invest 79:869-7). We have also demonstrated migratory and proliferative responses of human breast cancer cells to BSP, indicating it may stimulate primary tumour invasion by signalling through the $\alpha v\beta 3$ and $\alpha v\beta 5$ integrins (Sung et al, 1998. J Cell Phys 176:482-4). Although it was present in 70% of primary breast lesions examined, we did not detect BSP expression in a panel of human breast cancer (HBC) cell lines either in vitro or in vivo. To explore further possible functions for BSP in breast cancer invasion and metastasis, the full length BSP cDNA was transfected into the non-BSP-expressing MDA-MB-231-BAG cells under the control of the CMV promoter. 8 clones with differential expression levels of BSP and 3 vector control clones have been isolated and are being tested for adhesion, migration and proliferation. Depending on these in vitro studies, the clones will also be examined in vivo in models of breast cancer growth and metastasis. These studies directly test our hypothesis that BSP drives primary tumour progression. This work is supported by The Thomaiy Breast Cancer Research Fund and The Victorian Breast Cancer Research Consortium.

ENDOCRINOLOGY/PRECLINICAL AND CLINICAL 3: Steroid Hormones and Receptors

#879 EXPRESSION AND FUNCTION OF ANDROGEN RECEPTOR DURING INTERMITTENT ANDROGEN WITHDRAWAL IN VITRO. Z Culig, L Lambrinidis, G Bartsch, H Klocker, and A Hobisch, *Univ of Innsbruck, Innsbruck, Austria*

Intermittent androgen ablation is a therapeutical option for advanced carcinoma of the prostate which leads to improved quality of life of these patients. We have generated new prostate cancer cell sublines that mimic the situation in patients subjected to intermittent androgen withdrawal. In these cell lines, we have investigated androgen receptor (AR) expression and function. LNCaP-abl cells were generated from parental LNCaP cells after nine months propagation in steroid-deprived medium. They were then cultured in media with a) steroid-containing serum (FCS) and b) FCS and the synthetic androgen mibolerone. In these cases, LNCaP-R (a) and LNCaP-RA (b) cells were generated. AR protein expression was higher in LNCaP-abl cells than in any other subline (4.8 vs. 1.2 fmol/μg protein in the parental cells, 1.8 in LNCaP-R and 1.0 in LNCaP-RA cells). AR basal transcriptional activity was 30-fold higher in LNCaP-abl than in LNCaP cells. Reporter gene activity was induced by 10-fold lower concentrations of androgen in LNCaP-abl than in LNCaP cells. Bicalutamide evoked a 2-fold increase in reporter gene activity in LNCaP-abl cells thus indicating that it switches from an AR antagonist to agonist. Agonistic activity of bicalutamide was reversed in LNCaP-R and LNCaP-RA cells. Our results show that antagonist/agonist balance of bicalutamide is modulated by androgen during intermittent androgen ablation.

#880 INSULIN-LIKE GROWTH FACTOR BINDING PROTEIN-3 (IGFBP-3) MEDIATES 1,25-DIHYDROXYVITAMIN D GROWTH INHIBITION IN THE LNCAP PROSTATE CANCER CELL LINE. Bryan J Boyle, P Cohen, and D Feldman, *Stanford Univ Sch of Medicine, Stanford, CA, and UCLA, Los Angeles, CA*

$1,25\text{-}(OH)_2D_3$, the-primary active form of Vitamin D, is known to inhibit the growth of various cancer cell types, including prostate cancer. Although much work has been done to describe the mechanisms of action of $1,25\text{-}(OH)_2D_3$-induced growth inhibition, the exact mechanisms have not yet been defined. One way in which $1,25\text{-}(OH)_2D_3$ may act is by regulation of insulin-like growth factor binding protein-3 (IGFBP-3), which has been shown to inhibit prostate cancer cell growth. Here, using the LNCaP prostate cancer cell line, we show that $1,25\text{-}(OH)_2D_3$ is capable of inducing both IGFBP-3 mRNA production and protein secretion in a dose- and time-dependent manner. In addition, we demonstrate that exogenous IGFBP-3 causes growth inhibition in these cells. Finally, for the first time, we demonstrate that by blocking IGFBP-3 using either immunoneutralization or antisense oligonucleotides, the growth-inhibitory activity of $1,25\text{-}(OH)_2D_3$ is reversed in these cells. These findings suggest that induction of IGFBP-3 is an important and required component of the growth-inhibitory activity of $1,25\text{-}(OH)_2D_3$ in the LNCaP prostate cancer cell model.

#881 ROLE OF ESTROGENS IN NON-SMALL CELL LUNG CANCER. J M Siegfried, A L Gaither Davis, and C Gupta, *Univ of Pittsburgh, Pittsburgh, PA*

Increasing evidence suggests women are more susceptible than men to the adverse effects of tobacco exposure. One possible explanation is an involvement of estrogen as a proliferative agent in lung cancer. There have been conflicting reports of the presence of estrogen receptors on lung tumors, and the recent discovery of estrogen receptor β (ERβ) expression in lung tissue suggests either estrogen receptor α (ERα) or ERβ might mediate changes in cell proliferation in the lung. Here we report that in five non-small cell lung cell lines (one from a squamous cell tumor and four from adenocarcinomas), mRNA for ERα and ERβ was detected by reverse-g74 transcription polymerase chain reaction in all cases; the amplified product was more intense for ERα than ERβ. By western blot, protein for ERα was also detected in all cases. ERβ analysis by western blot is in progress. Normal bronchial epithelial cells rarely showed detectable ERα or ERβ by RT-PCR. Because stromal cells in endocrine organs contain high levels of ERs, we also examined lung fibroblasts (LFBs) for ERα and ERβ expression. We found a barely detectable amplification product for ERα and ERβ mRNA in five cultures of LFBs; no protein was detected by western blot. Expression of mRNA and protein was consistently higher in tumors compared to normal bronchial epithelial cells or LFBs. Estrogen at 1nM and 10 nM stimulated BrDU uptake into quiescent lung adenocarcinoma cells 8–10 fold using phenol-red free and steroid-free medium. These results suggest estrogens play a role in the promotion of lung cancer, analogous to their role in breast cancer. Expression of estrogen receptors may be part of the progression to malignancy in the lung.

#882 GENE EXPRESSION PROFILES WITH ACTIVATION OF ER-SERM COMPLEX. Anait S Levenson, K. M Svoboda, J. Horiguchi, and V. C Jordan, *Robert H Lurie Comprehensive Cancer Ctr and Northwestern Univ, Chicago, IL*

Tamoxifen and Raloxifene, referred to as selective estrogen receptor (ER) modulators (SERMs), have enormous therapeutic impact in breast cancer. The ER-SERM complex is a transcriptional regulator that controls patterns of gene expression. We developed a unique *in vitro* model system based on differential pharmacology of antiestrogens, which results from their interaction with the wtER or $mutant_{351}$ER in a breast cancer context. Using Atlas cDNA Expression Arrays (Clontech) we compared gene expression profiles in cells with wtER and $mutant_{351}$ER following treatment with estradiol (E2), 4-hydroxytamoxifen, raloxifene (Ral) and ICI 182,780 for 24hr and 48 hr. Although extensive similarity was noted between the gene expression profiles in different sets of experiments, there were numerous transcripts that were expressed at significantly different levels in cells 1) with wtER versus $mutant_{351}$ER; 2) untreated versus treated with E2; 3) treated with E2 versus SERMs versus ICI. Among the set of E2-responsive genes there were immediate-early genes including c-myc and Fra-1, growth factors, interleukins and transcription factors including IGFBP-1, IGFBP-3, IL-8 and ATF4. When comparing cells with wtER to cells with $mutant_{351}$ER, both treated with Ral, the set of differentially expressed genes included transcriptional activator hSNF2b, c-AMP-dependent protein kinase alpha-catalytic subunit and DB1. Comprehensive data analysis of results from various sets of experiments will allow us to understand how the altered gene expression patterns play a role in their ability to contribute to the agonist/antagonist properties of SERMs. Supported by ACS IL Division, Inc. and IDPH to A.S.L.

#883 OXIDATIVE/REDUCING AGENTS ALTER THE LIGAND BINDING AND DIMERIZATION OF ESTROGEN RECEPTOR (ER) ALPHA AND BETA. P. Farooque, Srivani Balabhadrapathruni, T. J Thomas, M. A Gallo, and T. J Thomas, *UMDNJ-Robert Wood Johnson Med Sch, New Brunswick/Piscataway, NJ*

Redox (reduction/oxidation) regulation through cysteine residues is known to modulate certain transcription factor functions. We examined the ligand binding activity and dimerization of recombinant ERα in the presence of agents that oxidize or reduce the sulfhydryl groups, by sucrose density gradient analysis. When ERα was treated with the oxidizing agent diamide, (250 to 1000 μM) in the absence of dithiothreitol (DTT), the ligand binding activity was lost. When ER was treated with diamide in the presence of 2 mM DTT, the peak of 7S dimer was increased by ~ 2-fold. Reduced form of glutathione caused stabilization of ER dimer at 250 to 500 μM range. With oxidized glutathione, a gradual decrease in dimeric ER was observed at 250 to 1000 μM concentration. With ERβ the dimeric form was stabilized with the addition of diamide in the presence of DTT. However, ERβ showed significant ligand binding as a monomer under strong oxidizing

conditions of diamide without DTT. These results indicate differential redox regulation of ERα and ERβ at the ligand binding and dimerization levels. Thus ER function appears to be influenced by redox regulation.

#884 ROLE OF PROGESTERONE RECEPTOR (PR) IN SUSCEPTIBILITY OF MOUSE MAMMARY GLAND TO PRENEOPLASTIC LESIONS. Robert T Chatterton, Esnar T Mateo, V. Craig Jordan, Rajendra G Mehta, and John P Lydon, *Baylor Coll Med, Houston, TX, Northwestern Univ Med Sch, Chicago, IL, and Univ of Illinois Coll Med, Chicago, IL*

The ability of DMBA to induce preneoplastic lesions (PEL) was studied in vitro in mammary glands of C57Bl/6 x 129SvEv mice bearing the progesterone receptor knockout (PRKO). Wild type (WT) and PRKO mice were pretreated daily for 15 days with 1.0 mg progesterone and 1.0 μg estradiol. Mammary glands were cultured for 10 days in a medium that, from previous work, promotes lobuloalveolar growth (LA) in Balb/c WT mice: Waymouth's + insulin, prolactin, aldosterone, and cortisol (IPAF), or an alternative medium: Waymouth's + insulin, prolactin, estradiol and progesterone (IPEPg). DMBA or dimethylsulfoxide vehicle was added for 24 h on day 3 of culture. All cultures contained only Waymouth's + insulin on days 10-24; this results in regression of hormone-dependent epithelial structures but not PEL. Results: IPAF medium: Glands of WT mice responded to DMBA with development of PEL. Glands of PRKO mice failed to develop LA but contained viable ductal epithelial cells based on proliferating cell nuclear antigen (PCNA) staining at 10 days; even in those treated with DMBA the epithelial structures regressed and were not viable at 24 days. IPEPg medium: At 10 days, the glands of both WT and PRKO mice exhibited intraductal PCNA-positive hyperplasia with some ductal budding but without LA. After DMBA, PCNA positive PEL were present in WT but not in PRKO mice at 24 days. Conclusions: In the absence of PR epithelial structures were resistant to the carcinogenic action of DMBA. This resistance applied to both alveolar and ductal structures. Thus, the absence of alveolar differentiation was not the basis for the protection from preneoplastic lesions afforded the PRKO animals by the PR knockout. (Supported by a grant from the Sage Foundation, Chicago, IL)

#885 EXPRESSION AND REGULATION OF THE ESTROGEN RECEPTOR IN RESPONSE TO TAMOXIFEN AND RELATED SERMS. Emma L Parrott, Paul S Jones, and Ian Nh White, *MRC Toxicology Unit, Leicester, United Kingdom*

The increased incidence of endometrial cancers in women treated with tamoxifen, an adjuvant treatment for breast cancer, has lead to the search for more tissue selective estrogen agonists. We have investigated the potential of the estrogen receptor (ER) to regulate the response to estrogenic compounds. Formalin fixed uterine sections from ovariectomised CD-1 mice, treated daily for 3 days with 17β-estradiol (2μg/kg/day) or tamoxifen (400μg/kg/day), were probed specifically for ERα or ERβ by *in situ* hybridisation. Predominantly, ERα and ERβ mRNA is localised in the epithelial cells of the lumen and glands. Compared to ovariectomised controls, treatment with 17β-estradiol and tamoxifen causes an increase in expression of ERα and ERβ mRNA in both epithelial and myometrial cell types. The co-expression of the two subtypes allows them to interact *in vivo*. This suggests that the relative level of the two receptors is a key component in the response of target cells to selective estrogen receptor modulators (SERMs). We have investigated control of ER degradation in endometrial and breast cell lines. Results show that proteasome-mediated ER degradation is ligand dependant.

#886 FUNCTIONAL ACTIVITY OF THE ANDROGEN RECEPTOR DEPENDS ON PROMOTER AND CELL CONTEXT. Miao Yang, Petra Neufing, Nicole Wilson, Nicole Moore, Grant Buchanan, Robert Matusik, and Wayne D Tilley, *Flinders Univ of South Australia, Adelaide, Australia, and Vanderbilt Univ Med Ctr, Nashville, TN*

In this study we report that promoter and cell context are important determinants of the functional activity of the androgen receptor (AR). Transient transfection studies using the AR negative prostate cancer cell line, DU145 revealed that DHT induces AR-dependent transcription from the androgen regulated prostate specific antigen (PSA) and mouse mammary tumor virus (MMTV) promoters, but not from the probasin promoter (PB). Both MMTV and PSA promoter driven reporter gene expression was regulated by DHT in a dose dependent manner in DU145 cells. In contrast, all three promoters are activated to a similar extent by DHT in PC-3 (transiently transfected AR) and LNCaP (endogenous AR) prostate cancer cells, the level of transactivation being 8-10 fold greater than in DU145 cells. AR protein was detected at comparable levels in LNCaP, and in transfected PC3 and DU145 prostate cancer cell lines, suggesting that the inability of DHT:AR complexes to transactivate the PB promoter in DU145 cells may be due to the absence of specific AR cofactors. AR variants identified in our recent studies of clinical prostate cancer also demonstrated different transactivation activities with each of the promoters in PC3 cells. We conclude that cell and promoter context plays an important role in regulating the androgen signaling axis in prostate cancer cells. Furthermore, differential regulation by AR variants of genes controlling the growth of prostate cancer cells may contribute to disease progression.

#887 DEHYDROEPIANDROSTERONE (DHEA) STIMULATES PROLIFERATION OF MCF-7 CELLS AFTER BEING METABOLIZED TO ESTROGENS. Martina Schmitt, Klaus Klinga, and Doris Mayer, *Deutsches Krebsforschungszentrum, Heidelberg, Germany, and Universitaetsfrauenklinik, Heidelberg, Germany*

DHEA, a steroid of the adrenal cortex serves as a precursor for the synthesis of androgens and estrogens. DHEA shows mitogenic effects in estrogen receptor (ER)-positive breast cancer cells. Our aim was to determine, whether or not DHEA requires metabolism to potent hormones in order to stimulate cell proliferation, and if so which metabolites mediate this effect. Proliferation of the ER-positive breast cancer cell line MCF-7 was quantified by BrdU-ELISA. 17β-estradiol (E2)-content of the conditioned media was determined by immunoassay after extraction with organic solvents and partial purification by thin layer chromatography. In MCF-7 cells stably transfected with an ERE-luc plasmid (MELN cells), ERE-dependent gene expression was quantified by measuring luciferase activity. DHEA-induced stimulation of cell proliferation could be abolished completely by the anti-estrogen ICI182,780. After incubation with 100 nM DHEA, E2-concentrations >100 pM in the medium were found sufficient for the mitogenic effect, whereas 5Δ-androstenediol, another compound binding to ER, was not produced in amounts required to stimulate cell proliferation. The aromatase inhibitor 4-hydroxyandrostenedione (OH-A) also prevented proliferation stimulation; in parallel E2 content declined. ERE-dependent transactivation was maximally induced by 100 nM DHEA after 4 but not after 2 days, implicating the need of metabolic transformation. The DHEA-induced transactivation was also reversed by OH-A. We conclude that DHEA acts as mitogen in breast cancer cells only after being metabolized to estrogens.

#888 EFFECT OF CADMIUM ON ANDROGEN RECEPTOR REGULATION IN PROSTATE CANCER. Adriana Stoica, James H Voeller, Edward P Gelmann, Elly G Stoica, and Mary B Martin, *Georgetown Univ, Washington, DC*

Androgens play a central role in tumor development. They are thought to promote tumor growth in androgen-responsive cells by androgen receptor (AR)-mediated effects. The role of the heavy metal cadmium (Cd) in AR regulation was investigated in the human prostate cancer cell line LNCaP. Cadmium (10^{-6} M) stimulated the growth of cells 2.7-fold after 8 days and the population of cells in S+G2M phases increased 3-fold after 4 days of metal treatment. Cadmium decreased the concentration of AR protein by 48% as measured by Western blot and ligand binding assay. RNase protection showed that Cd induced a parallel decrease in AR mRNA (50%). Two androgen-regulated genes, prostate specific antigen (PSA) and the homeobox gene NKX 3.1 increased 5-fold and 3- fold, respectively. All these effects were blocked by the antiandrogen casodex (10^{-5}M). Cadmium also stimulated an androgen response element in mouse L cells, which endogenously express wild-type AR, stably transfected with a MMTV-luciferase reporter and in COS-1 cells transiently co-transfected with wild-type AR and an MMTV-CAT reporter. Scatchard analysis demonstrated that cadmium binds to the AR with high affinity, Kd=0.95+/-0.054 nM (n=3, r=-0.796). In chemically orchiectomized male Wistar rats, low doses of Cd (10-20 ug/kg body weight) increased the prostate wet weight by 1.8-2.5-fold and the weight of the seminal vesicles by 1.7-fold. The increase in wet weights was blocked by the antiandrogen cyproterone acetate (50 mg/kg body weight), suggesting androgenic effects of Cd in animals. Taken together, these data suggest that the effects of cadmium are mediated by AR independent of androgens. By activating AR, human exposure to cadmium may increase the risk for prostate cancer.

#889 INVOLVEMENT OF PROGESTERONE RECEPTOR (PR) IN THE ORGANIZATION OF BASEMENT MEMBRANE INTEGRITY IN MAMMARY GLANDS. Marina Simian, Adam Vigil, Mina J Bissell, and Gopalan Shyamala, *Lawrence Berkeley Lab , Univ of CA, Berkeley, CA*

PR is a member of the superfamily of nuclear receptors that mediates the action of progesterone and is essential for mammary development during pregnancy. PR exists in two molecular forms, the A and B forms, and in transgenic mice carrying additional A form (referred to as PR-A transgenics), there is abnormal mammary development accompanied by a disruption of basement membrane organization, as revealed by discontinuous laminin staining, suggesting that PR signalling may play a role in the organization of basement membrane during mammary development. It is well established that the integrity of the basement membrane in the mammary gland is dictated by the levels of matrix metalloproteases (MMPs). Therefore, as a first step towards understanding the potential involvement of PR in the organization of the basement membrane, we compared the MMP levels in tissue extracts of wild type and PR-null mutant mice. These studies demonstrated a significantly lower level of MMP-2 in extracts of PR-null mutant mice. Furthermore, when wild type ovariectomized mice were treated with estrogen and progesterone, with the increase in PR levels, there was also an increase in MMP-2 activity as compared to either untreated mice, or mice treated only with estradiol. Thus these studies also revealed a positive relationship between PR levels and MMP-2, and the requirement for progesterone to modulate this activity. We propose that nuclear signalling by PR can influence the organization of the basement membrane by exerting a positive effect on protease activity. (Supported by grants from UCBCRP (4JB0142) and DOE (DEC-AC03-76SF00098)).

#890 ERβ MRNA EXPRESSION IN HUMAN COLON ADENOCARCINOMA CACO-2 CELLS. Martha Campbell Thompson, Jeanette Lynch, and Bhavna Bhardwaj, *Univ of Florida, Gainesville, FL*

Epidemiological studies show that colon cancer incidence and mortality rates are lower in females compared to males and that estrogen replacement therapy reduces colorectal cancer risk in postmenopausal women. The molecular mech-

APPENDIX K

The 1998 Annual Retreat on Cancer Research in New Jersey

May 29, 1998

GALLO AWARD

Presented by

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Srivani Balabhadrapathruni

Research Titled

Genistein Treatment Causes G_2/M Cell Cycle Arrest and Cyclin B1 Accumulation in Breast Cancer Cells: A Possible Mechanism for the Preventive Action of Genistein

T. S. Ravikumar, MD
Associate Director for Education and Training
The Cancer Institute of New Jersey
Co-Chair, The 1997 Annual Retreat on Cancer Research in New Jersey

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